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Physicochemical Properties and Molecular Structure Determination of Glycogen From Anthonomous Grandis, Boheman.

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PHYSICOCHEMICAL PROPERTIES AND MOLECULAR STRUCTURE DETERMINATION OF GLYCOGEN FROM *ANTHONOMUS GRANDIS* BOHEMAN

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ABSTRACT

Although studied extensively in many animals, very little is known concerning the structure of insect glycogen. The object of this study was to determine the physical and chemical properties and the molecular structure of glycogen from the boll weevil, Anthonomus grandis Boheman.

Glycogen was extracted with 5% trichloroacetic acid (TCAe.) and with cold chloroform-glycine buffer (c.w.e.). Carbohydrate content of the TCAe. glycogen was 66.3% with 6.3% protein, 7.7% ash, and 10.4% moisture. The c.w.e. glycogens averaged 97.0% carbohydrate.

Similar infra-red spectra were obtained from TCAe. and c.w.e. glycogen and also from TCAe. and c.w.e. glycogen complexes with iodine-iodide when analyzed in the visible region. The optical rotation of TCAe. larval glycogen was +200.0°, and was the highest (by 6°) reported for insect glycogens. Opalescence of c.w.e. glycogens prevented measurement of its optical rotation.

Ultracentrifugal sedimentation analyses showed that, with the exception of glycogen from boll weevil eggs, c.w.e. glycogens had low (200 svedbergs) and high (1200 svedbergs) molecular weight regions. Trichloroacetic acid extracted larval glycogen, on the other hand, had only a low (100 svedbergs) molecular weight component and in this respect was similar to a potassium hydroxide extracted rabbit liver

glycogen standard (150 svedbergs). Corrected sedimentation patterns were not obtained for c.w.e. egg glycogen because of a fraction with a molecular weight too high for the IBM computer to correct accurately. Boll weevil egg glycogen has the highest (weight average; based on contact prints from the ultracentrifuge) molecular weight of all glycogens yet analyzed.

After sulfuric acid hydrolysis, all boll weevil glycogens (TCAe. and c.w.e.) yielded glucose, with no other sugar being detected by thin-layer or gas liquid chromatography.

After incubation with β-amylase, using a new technique, i.e., isolation and identification of maltose after elution from a column of Sephadex G-200, the smallest β-amylolysis limit dextrins yet defined for glycogen were obtained. This indicated either longer exterior chains for boll weevil glycogen or a larger number of exterior chains than usually are present in glycogen extracted from other organisms.

The limit of phosphorylase b action on boll weevil glycogens varied with the extraction procedure, with the TCAe. larval sample being degraded more (39.6%) than either the c.w.e. egg or adult glycogens (30.4 and 26.5%, respectively).

When the average exterior chain length was calculated by enzymic assay, the results with β-amylase indicated an average exterior chain length of 8.5 glucose units; the results with phosphorylase b gave an average exterior chain length of 7.8 units. The average interior chain length by these two enzymic methods were 3.2 and 4.2 units, after cleavage by β-amylase and phosphorylase b, respectively.

Average chain lengths were also determined by methylation, hydrolysis, silanization, and gas liquid chromatography of the hydrolytic products. This study has shown, for the first time, that polysaccharides can be methylated directly with one set of methylating reagents. The average chain length and linkages present in the glycogen molecule were determined from the ratio of tetra:tri: dimethylglucopyranoses present in the hydrolyzate.

Cold-water extracted egg and larval, and TCAe. larval glycogen, when analyzed by the methylation method, had chain lengths of 11.0, 11.9, and 11.8, respectively. The high percentage of 2,3,6-trimethylglucopyranose obtained (82%) indicated that the principal linkage and configuration between glucose units was α-1,4.

INTRODUCTION

Complex chemical structures of polysaccharides have long been of interest to biochemists and physiologists, but a detailed study of insect glycogen is not yet available. Although glycogen is a major reserve of insects, only brief and fragmentary data have been reported for insect glycogens (Table 1).

Glycogen is the collective name given to a group of highly branched polysaccharides isolated from animal cells and microorganisms, which conform to the empirical formula, $(C_6H_{10}O_5)_n$. Glycogen has also been characterized by its high dextrorotation ($+179^{\circ}$ - $+233^{\circ}$), its red-brown coloration with iodine, and opalescence of its water solution. Complete acid hydrolysis yields glucose quantitatively and its high average molecular weight ($1\text{-}10 \times 10^6$) makes glycogen the largest naturally occurring polysaccharide. "Polymolecular" and "polydisperse" are terms frequently associated with the molecular size of glycogen. Its isolation from biological material, independent of the extraction procedure employed, is based exclusively on the insolubility of glycogen in ethanol.

Analysis of the chemical composition of numerous insects show that fat and glycogen constitute the principal food (and therefore energy) reserves. It is difficult, however, to determine the values attributed to "glycogen" in the early literature on insects. The data

Table 1. Comparison of Properties Associated with Insect Glycogens and Mammalian Glycogen.

	Op. Rot.	Acid Hydrolysis
Insect Glycogens		
Hyalophora cecropia[1/]	+184°	Glucose + Maltose
Gasterophilus [2/]	+192.6°	-
Drone larvae [3/]	+191°	-
Aphid [4/]	+194°	Glucose
Horse Bot Fly [5/]	-	Glucose
Bombyx mori [6/]	-	Glucose
Bombyx mori [7/]	-	Glucose
Mammalian Glycogen		
Rabbit Liver	+178°	Glucose

1/ Bade and Wyatt, 1962
2/ von Kemnitz, 1916
3/ Abdel-Akher and Smith, 1951
4/ Loring and Pierce, 1943
5/ Levenbook, 1950
6/ Chino, 1957
7/ Zaluska, 1959

are complicated by the omission of extraction procedures used by the respective authors. Evans (1932) reported that a white non-reducing substance, obtained from the sheep blow fly, Lucilia sericata, was insoluble in 70% ethanol, gave an opalescent water solution and a reddish-brown color with iodine. However, on acid hydrolysis this glycogen-like material did not yield reducing sugars. The method of extraction was not reported, but it was concluded from several tests that the glycogen-like material was not protein. A similar material was isolated from the desert locust, Schistocerca gregaria, by Howden and Kilby (1960). The material behaved on assay as glycogen but apparently was not true glycogen. When the glycogen-like material was subjected to alkaline digestion followed by acid hydrolysis, the increase in reducing sugar amounted to only 3-4% of the starting material. On the addition of an iodine solution to the material, a yellow color was produced (as opposed to reddish-brown) that had a maximum absorption around 400 mμ. The acid hydrolyzates were subjected to a wide variety of reducing-sugar indicators, but no reducing substances were detected. Levenbook (1951) isolated a glycogen-like material from the bot fly, Gasterophilus intestinalis, by alkaline digestion, but concluded that the extracted material was not true glycogen.

Interestingly, the field of insect glycogen analysis is neither original nor singular in its descriptions of glycogen-like materials. Since the suggestion of Meyer and Press (1941) that a glycogen-type polysaccharide was isolated from corn, most of the extensive work on characterization of true glycogens have included

samples of this polysaccharide. Later, Meyer and Fuld (1949) stated that "the sugar from Zea mays has been studied to the point of its constitution; molecular weight, and degradation by β-amylase. It is indistinguishable from animal glycogen." Morris (1944) characterized this glycogen-like material by its cleavage with β-amylase and concluded that this corn "glycogen" (degraded only 20% by β-amylase) was more highly branched than defined animal glycogens. Most workers now classify this material as "phytoglycogen."

Very little information is available on insect glycogen. Von Kemnitz (1916) reported that glycogen from Gasterophilus intestinalis had an optical rotation of $[\alpha]_D^{20} = +192.6^\circ$. Two species of aphids were trichloroacetic acid (TCA) extracted for glycogen by Loring and Pierce (1943); the samples gave the same rate of hydrolysis with 0.5N sulfuric acid as did a rabbit liver glycogen standard. Only glucose was identified in the hydrolyzates. The glycogens from both species, Macrosiphum pisi and Aphis brassicae, gave the characteristic color with iodine and their water solutions gave $[\alpha]_D^{25} = +194^\circ$. Levenbook (1950) isolated larval glycogen from the horse bot fly, Gasterophilus intestinalis, which contained only glucose as the component monosaccharide. An extensive analysis of 37 glycogens from numerous sources was carried out by Abdel-Akher and Smith (1951). Included in their work was glycogen isolated from drone larvae, which had an optical rotation of $+191^\circ$ and upon chain length analysis (by periodate oxidation of 0.55g) gave an average chain length of 12 glucose units. All values reported by them were between 10-14 glucose units and it

was concluded that all glycogens are composed of approximately 12 units. Moulinier (1956) adapted the Folin and Wu (1920) method of glucose analysis to the determination of glycogen in the eggs of Bombyx mori. Although not specifically stated, it is assumed that glucose was obtained by acid hydrolysis. While studying carbohydrate metabolism in the silkworm, Bombyx mori, Chino (1957) isolated glycogen with hot TCA. The glycogen was subjected to hydrolysis with 1N hydrochloric acid and only glucose was found in the hydrolyzate. More recently, Bade and Wyatt (1962) characterized glycogen from the cecropia silkworm by electrophoresis of the polysaccharide in borate buffer. Acid hydrolyzates were subjected to paper chromatography in two solvent systems and a predominance of glucose was obtained. A small amount of a disaccharide was detected by the spray reagent (ammononical silver nitrate) and was thought to be maltose. The optical rotation of a 0.5% solution of cecropia silkworm glycogen was $+184^{o}$.

Several excellent reviews are available on the synthesis, degradation, and molecular structure determinations of animal glycogens from many sources (Manners, 1962; Manners, 1957; Greenwood, 1956; Greenwood, 1952). More recently, 1964, the CIBA Foundation symposium has published "Control of Glycogen Metabolism," an extensive review on present conceptions of glycogen structure, isolation, synthesis, and degradation (in vivo and in vitro).

Extraction procedures for the removal of glycogen from biological tissues have undergone extensive changes. The standard

technique for the extraction of glycogen for nearly 50 years was that described by Bernard (1857) and modified by Pflueger, as cited by Orrell, et al. (1964). This method involved prolonged heating of the tissue in strong alkali (30% potassium hydroxide). An additional modification by Somogyi (1934), digestion with 60% alkali at 100°C for three hrs., has been used extensively to prepare nitrogen, phosphorus, and salt-free glycogen. Stetten et al. (1956) have developed a method of isolation using cold trichloroacetic acid (TCA) similar to that first reported by Sahyun and Alsberg (1930). Extraction of glycogen has also been accomplished by homogenization of the tissue in hot water (Greenwood and Manners, as cited by Manners (1957)), in dimethyl sulfoxide (DMSO, Whistler and BeMiller, 1962) and in cold water (Lazarow, 1942).

Recently Bueding and Orrell (1965) developed a cold-water extraction procedure which, according to Orrell et al. (1964), yields a product which approaches more closely the state of glycogen in the living cell. A recent report of BeMiller (1965) presented a critical review of these extraction methods and their products. According to the report, alkaline extraction causes extensive degradation and TCA extraction yields a somewhat hydrolyzed product with some degree of polydispersity. Extraction with DMSO apparently gives a much higher molecular weight glycogen with no evidence of polydispersity, but in low yields; the aqueous preparations produce the highest molecular weight material but with extreme polydispersity. It is also pointed out by BeMiller (1965) that hot water, acid, and alkali should be avoided if "native" glycogen is desired.

This corresponds to the instruction of Orrell *et al.* (1964) that studies concerned with glycogen structure and metabolism must be carried out with the milder, cold-water extraction procedure.

The optical rotation of glycogen was first recorded by Harden and Young (1902) and values obtained ranged from $+179^{\circ}$ to $+233^{\circ}$ depending on the source of the sample. Their observations have been the most enduring of reported glycogen data and, most often, is the primary method of identification. Recent improvements in extraction methods, especially the milder procedures, have produced such highly opalescent solutions that 1% aqueous solutions are unsuitable for polarimetric measurements (Manners, 1957).

The iodine-binding power of glycogen has frequently been used to distinguish between amylose, amylopectin, and glycogen. Swanson (1948), studying the structure of polysaccharides, reported the iodine colors obtained for branched and phosphorylase-synthesized polysaccharides. Illingworth and Cori (1952) reported that human glycogen, exposed to a solution of iodine in potassium iodide, exhibited maximum absorption at 470mμ . Whelan and Bailey (1954) used the iodine-iodide medium to follow the action pattern of potato phosphorylase. Archibald *et al.* (1961) reported maximum absorption values of 455-485mμ for rabbit liver glycogen samples. Manners (1962) reported the region of maximum absorption for rabbit liver glycogen in iodine-iodide to be 460mμ . Recently, Ghosh and Preiss (1965) showed the absorption spectra of the iodine complex for rabbit liver and *Arthrobater* glycogen to be 430 and 420mμ , respectively.

Absorption of glycogens in the infra-red has been studied extensively by Barker et al. (1954). They stated that the characteristic band of absorption was in the frequency range 730-960cm^{-1} and that this region produced three peaks (928 ± 3, 839 ± 3, 760 ± 2 cm^{-1}). It was shown that the peak at 839cm^{-1} was exhibited by all carbohydrates containing the α-D-glucopyranose structure, while those appearing at 760 and 928cm^{-1} were characteristic of only α-1, 4 linked glucans.

Chemical methods of molecular weight determination (macromolecular compounds) have been described by several workers. However, these methods must be combined with structural analyses, and at best, yield only an estimate of the molecular weight. Direct measurements of the molecular weight of glycogens have been successfully measured by osmometry, light scattering, sedimentation and diffusion, and least successfully by ultramicroscopy. Kratohvil (1964) has discussed the general problem of the accurate determination of molecular weights of macromolecules. The results of osmotic pressure and light scattering measurements are often uncertain and confusing. The molecular weights or rabbit liver and rabbit muscle glycogen were determined by Oakley and Young (1936) to be 1.2-2.3 x 10^6 and 0.7-1.8 x 10^6, respectively. A special form of a low pressure osmometer was constructed which required a temperature control of ±0.005°C. They found that ions, particularly Ca^{++} and Cl^-, had a profound influence on the osmotic pressure of the glycogen solutions. The major objection to the use of osmometry has been the expression of the data yielded by this technique. Since the number of molecules of solute are counted, the molecular weight is obtained as a number average (as opposed to weight

average by sedimentation data) which requires that the macromolecules have a very narrow molecular weight distribution. Bridgman (1942) reported conflicting molecular weight data for glycogen. On analysis by end-group reactions, a small molecule was reported to have been indicated, but osmotic pressure data on the same sample indicated a high molecular weight (3.5×10^6).

The most serious problem, that makes light scattering of limited value as a means of determining molecular weight, arises from the presence of small (partially soluble) solute material in the sample. Presence of such solutes leads to such grossly distorted diagrams that the results are most often too high. Dandliker and Kraut (1956) and others have reported a ultraclarification technique involving the centrifugation of the solutions in specially constructed light scattering cells. However, even with this technique and prolonged ultracentrifugation, the results are not satisfactory and the measured molecular weights are much too high. Light scattering techniques were used by von Staudinger (1948) to obtain the molecular weights of rabbit muscle (1.5×10^6) and rabbit liver (23×10^6) glycogens. Elias (1958) has reported a range of molecular weights for native dextran materials by light scattering.

For determination of molecular size and weight, sedimentation analysis has proved the most effective of the methods available. Mystkowski (1937) probably was first to use sedimentation analysis as a means of estimating the molecular weight of glycogen. When glycogen was extracted with a 4% aqueous trichloroacetic acid (TCA) solution, a polydisperse sedimentation pattern was obtained.

Mystkowski (1937) attributed the high molecular weight and polydisperse pattern obtained to aggregates of various sizes. Chargaff and Moore (1944) estimated the molecular weight of TCA extracted glycogen by sedimentation and diffusion. Glycogen was separated from the supernatant at 31,000 x g; the sedimentation velocity was reported as 170 svedbergs and the diffusion coefficient was 0.925×10^{-7}, which together gave an average molecular weight of 12.1×10^6. Bell et al. (1948) extracted rabbit liver glycogen by two methods (hot alkali and water) and reported that the alkaline extraction did not affect the sedimentation pattern. Their values were 4.4×10^6 and 4.3×10^6 for rabbit liver glycogen extracted with 30% KOH and 3% TCA, respectively, and were obtained by the calculations of Svedberg and Pedersen, as cited by Bridgman (1942). However, these mean molecular weights were based on the theory that the measured diffusion constant can be used to evaluate the frictional resistances to sedimentation and may be as low as 2×10^6 if the glycogen molecule is spherical. Von Staudinger (1948) and Bell et al. (1948), working independently, found that glycogens are extremely polymolecular and can be fractionated. Stetten et al. (1956) reported that appreciably greater degradation of glycogen resulted from exposure to hot KOH than to cold TCA. Average molecular weights ranged from $11 - 80 \times 10^6$ for TCA extracted glycogen and from $2 - 6 \times 10^6$ for KOH extracted material. They determined average molecular weights by light scattering and found that glycogen from rabbit liver or muscle was highly polydisperse. Stetten et al. (1958) reported the effects of KOH and TCA on rabbit liver glycogen extracted by these reagents. They observed a continuous decline in the molecular

weight of glycogen dissolved in hot KOH; the decline was more rapid in 1N than in 10N alkali and proceeded faster under oxygen than under nitrogen. The TCA extraction of glycogen yielded a product of far higher molecular weight than that obtained by digestion with alkali. The molecular weight of glycogen declined rapidly in TCA at room temperature and more slowly at 0°C. Sedimentation data obtained showed regions of high molecular weight which, according to Stetten et al. (1958), were in excess of 50×10^6. Stetten and Katzen (1961) have also reported that KOH extraction resulted in a rapid reduction in molecular weight and produced degradative changes of the glycogen molecule.

According to Orrell et al. (1964), extraction of glycogen with either KOH, TCA, or hot water considerably reduced the average molecular weight of samples from Ascaris, Fasciola hepatica, and Hymenolepis diminuta. The TCA and hot water extracted glycogens showed these degradative effects although not to the same extent as hot alkali. They have shown that frequently two regions of molecular weight are obtained for cold water extracted materials, as determined by their sedimentation-distribution differences. The first region corresponds to a lower molecular weight material (weight average, about 50×10^6) and the second region to a heavy molecular weight material (approximated as 450×10^6).

Molecular weight determinations (based on molecular size) have been performed by Husemann and Ruska (1940). They used a commercially available glycogen of a relatively low molecular weight (approximately 1×10^5); similar studies have shown (Meyer and Jeanloz, 1943) that the relationship used in ultramicroscopy did not hold for glycogens

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with molecular weights of 2-6 x 10^6 and greater. This technique also required that the material be homogeneous and of a defined particle size. However, the recent literature (a review was reported by Manners, 1957) shows that glycogens are inhomogeneous and also vary in shape.

Complete acid hydrolysis of glycogen yields glucose quantitatively and this has been used by many workers as a quantitative assay. Partial acid hydrolysis yields a mixture of sugars, other than glucose; maltose and maltotriose will arise from the linear portions of the chains. Unhydrolyzed branch points will produce other sugars; isomaltose provides evidence for α-1, 6-glucosidic linkages in the molecule. Thompson et al. (1954) reported six di- and trisaccharides were formed, not from glycogen hydrolysis, but from exposure of D-glucose to 0.082N HCl for 10 hours at 98°C. They identified these products as their acetate derivatives (formed with acetic anhydride, after exposure to the acid) and attributed the changes to acid reversion. Wolfrom et al. (1951) acid hydrolyzed rabbit liver glycogen and separated the acetylated derivatives of the hydrolytic products. Acid hydrolysis was determined to have been 75% complete and 1.8% β-isomaltose octa-acetate was obtained. Bacon and Bacon (1954) obtained isomaltose by carbon-Celite chromatography of a partial acid hydrolyzate of rabbit liver glycogen.

The complete acid hydrolysis of glycogen was obtained by Good et al. (1933). They hydrolyzed small amounts (less than 1 mg) of glycogen with 1N sulfuric acid at 100°C for 2 - 2.5 hours.

Hydrolysis of glycogen by β-amylase results in 45 ± 5% conversion to maltose (Bell and Manners, 1952). Barker et al. (1950) reported a method of glycogen incubation with β-amylase while studying the enzymic degradation of starch. Myrbäch (1948) reported the action of β-amylase as a stepwise hydrolysis of alternate linkages in a chain of α-1, 4-linked D-glucose residues, liberating maltose. The action begins at the non-reducing end of the chains and ceases when linkages other than α-1, 4 are encountered. The action of β-amylase on glycogen is confined to the exterior portions of the chains; the products are maltose and a high molecular weight limit dextrin (L.D.) Mayer and Larner (1959) showed that β-amylase splits the C-1-O bond rather than O-C-4 bond. The simplest mechanism reported for this action is a single-displacement reaction in which the water molecule attacks the potentially aldehydic carbon atom from the backside, causing an inversion of configuration at C-1. Thoma and Koshland (1960) have reported another theory on β-amylase action in which the conformation of the enzymic protein changes and catalytic groups are brought into alignment for enzymic reaction. They report the probability that the reaction involves one sulfhydryl group, one imidazole residue, and a carboxyl group at the active center, with at least three glucose residues of the substrate participating in the reaction.

The average exterior chain length can be determined for glycogens by digestion with β-amylase. Computation of the exterior chain length, however, requires a value for the outer stubs of the β-amylolysis limit dextrin. These outer stubs have been estimated to be from 1.0 to 2.5 glucose units in average length. Sillen and Myrbäch

(1943) reported the average number of glucose units remaining after cleavage by β-amylase to be 1.0. Meyer (1943) also calculated the length of the outer stub; his calculations suggested that the number of glucose units remaining in the outer stub varied between 1.0 - 2.0, and the average was 1.5 units. Later, French (1957) reported average outer chains of 1.0 - 3.0 glucose units in length and the average was reported as 2.0. However, the most frequently used value for the glucose units in the outer stub (2.5) was reported by Manners (1957). His values were 2.0 - 3.0, and were calculated excluding the glucose unit that is triply branched at the branching unit. These variations in outer stub size following cleavage by β-amylase have a direct effect on the interior chain length determinations using this enzyme. Thus, for a glycogen of an average chain length of 11 glucose units, 45% degraded by β-amylase (outer chain removed = 5.0 units), and 1 triply branched glucose unit. Therefore, the interior chain length will vary with the length of this residual stub. Since the outer stub varies between 1.0 - 2.5 units, the interior chain length for this glycogen will vary from 2.5 - 4.0 glucose units.

Evidence from partial acid (French et al., 1952) or enzyme (Peat et al., 1952) hydrolysis of the limit dextrin and from action of β-amylase on low-molecular-weight model substrates (Summer and French, 1956) is in best agreement with an average stub length of 2.0 glucose units.

Phosphorylases have been highly purified from many animal sources and some have been crystallized. Most phosphorylases exist in more than one form; an active form denoted a and an inactive form

designed by b which may be converted to a by adenylic acid. Phosphorylase a acts on glycogen from the non-reducing glucose residue in the exterior chains, in a manner similar to the attack of β-amylase. However, with phosphorylase (Cori and Larner, 1951) D-glucose residues are removed from the exterior chains as glucose-1-phosphate and action ceases near the branch points. The hydrolytic products are glucose-1-phosphate and a limit dextrin. Cori and Larner (1951) and others have reported that the average attack on glycogens by muscle phosphorylase is 30-50%. Muscle phosphorylase, however, does not degrade all exterior chains to the same extent (Illingworth et al., 1952). By contrast, potato phosphorylase, under similar conditions, gives about 10% degradation of glycogen.

The limit of action of phosphorylase is not known with certainty and results of phosphorylase degradation are confusing. The limit had been reported to be one glucose unit removed from the triply branched glucose residue (Cori and Larner, 1951). Walker and Whelan (1960) challenged the theory of Cori and Larner (1951) concerning the structure of the phosphorylase limit dextrin. The structure proposed by Walker and Whelan (1960) for the limit dextrin produced by the exhaustive action of phosphorylase, contained four glucose units in the outer stub. To gather adequate information to test the two theories, Hers et al. (1964) selectively labeled (with $U^{14}C$, glucose-1-phosphate) the peripheral position of the phosphorylase limit dextrin. This labeled limit dextrin was subjected to degradation by β-amylase. If the limit dextrin had the Cori and Larner structure, 50% of the radioactivity would be removed by β-amylase;

if the structure were that proposed by Walker and Whelan, all the radioactivity would appear in the maltose. Hers et al. (1964) reported that 100% of the radioactivity was liberated by β-amylase. They concluded that at least 40% of the phosphorylase stubs were four glucose units long. In order to explain the liberation of free glucose from the phosphorylase limit dextrin, Walker and Whelan (1960) assumed that their structure was converted to the Cori-Larner molecule by an enzymic transglucosylation. Brown and Illingworth (1962) showed that such an enzyme (1, 4 → 1, 4-glucantransferase) was a minor contaminant of the purified amylo-1,6-glucosidase used by Cori and Larner (1951) during the complete degradation of glycogen for chain length analysis. The transferase, named by Brown and Illingworth (1962) as oligo-1, 4 → 1, 4-glucantransferase, has been shown to transfer three glucose units (maltotriose) at a time. Interestingly, this is exactly the segment whose removal is required in a one-step conversion of the Walker-Whelan into the Cori-Larner structure. Additional evidence of this mechanism has recently been presented by Abdullah and Whelan (1963) and by Brown, Illingsworth, and Cori (1963).

The enzymic debranching of glycogen by rabbit muscle enzymes was first described by Cori and Larner (1951). Two processes were involved, first the phosphorolysis of glycogen to the limit dextrin and second the hydrolytic action of amylo-1,6-glucosidase on the triply branched glucose residue, rendering the molecule susceptible to further phosphorolysis. The combined action of the two enzymes caused complete conversion of glycogen to a mixture of glucose-1-phosphate and glucose, the proportion of the latter being equal to the proportion of branch

points (Illingworth, Larner, and Cori, 1952). Bueding and Hawkins (1964), using the procedures reported for this double-enzyme system, developed a method for the microdetermination of glycogen. Their method yielded data for the phosphorylase limit dextrin (by the action of phosphorylase alone), the percent branch points (by the combined action of phosphorylase and amylo-1,6-glucosidase, containing transglucosylase activity), the average chain length and the average exterior and interior chain lengths.

Chemical determination of the average chain length can be accomplished by the acid hydrolysis of fully-methylated glycogen, isolation and identification of the hydrolytic products. On hydrolysis, a fully methylated, branched α-1, 4-glucan should give a mixture of 2, 3, 4, 6-tetra-, 2, 3, 6-tri-, and one or more di-O-methylglucopyranoses, equimolar with the chain length. The configuration of methyl groups on the di-O-methylglucopyranose will be indicative of the inter-chain linkage at the branch points.

Analysis of mixed methyl ethers of sugars was formerly performed by fractional distillation (Haworth and Machemer, 1932) of the methyl glucosides or by chloroform-water partition of tetra-O-methylglucopyranose from the mixture (Hirst and Young, 1938). A colorimetric method (Bartlett et al., 1951) was reported in which the methylated glucopyranoses were separated by paper chromatography, eluted with hot methanol, and reacted with a solution of aniline hydrogen phthalate. Barker et al. (1950) reported the separation of methylated glucopyranoses by partitioning on silica with chloroform, chloroform-butanol (9:1), and acetone. They observed a mono-methyl

derivative of glucose and an unsubstituted glucose in the hydrolyzate, which was methylated by alternately adding (at -70°C) sodium iodide and methyl iodide. Hirst et al. (1949) hydrolyzed a fully methylated glycogen sample with 4% methanolic hydrogen chloride in a sealed tube at 100°C. Acid hydrolysis was performed by the addition of 4% aqueous hydrochloric acid at 100°C for three hours. The hydrolyzate was neutralized with solid silver carbonate, filtered, and passed through a bed of Amberlite IR-4B resin. The hydrolytic products were separated by paper chromatography and the chromatograms were developed with ammononical silver nitrate. The glycogen yielded a ratio of 1.0:8.2:1.1 (tetra:tri:dimethylglucopyranose), plus a small amount (10.6%) of another di-O-methylglucopyranose (other than 2, 3) and some (2.5%) mono-methylglucopyranose.

Experimentally, it seems impossible to methylate glycogen completely despite repeated treatment with various methylation reagents (Bell, 1948). Consequently, hydrolysis of methylated glycogen (12-unit) yields data in the molecular ratio of approximately 1:10:1 (Bell, 1937).

Bacon et al. (1944) improved the methylation of glycogen by forming the fully acetylated derivative followed by deacetylation and methylation of the molecule.

A method for methylating disaccharides was reported by Kuhn et al. (1955) and later modified (1960). Methylation was carried out at 20°C for 12 - 18 hours in a stoichiometric mixture of methyl iodide and silver oxide; the reaction was performed in a N, N-dimethylformamide solution of the disaccharide. The methylated disaccharide was extracted by partitioning with chloroform-potassium cyanide solution (1%).

Recently, gas chromatographic methods have been used to separate and quantitate mixtures of hydrolytic-products from fully methylated polysaccharides (Bishop and Cooper, 1960; Bishop *et al.*,1960; Perila and Bishop, 1961). These investigators were determining the constitution of glucomannans and cell wall polysaccharides by methylation of the molecules in a mixture of dimethyl sulfate and sodium hydroxide, followed by gas-liquid chromatography of the hydrolytic products.

Sephton (1964) reported another method for the determination of the hydrolytic products of a fully methylated, hydrolyzed xylan by silanization of the methylated pentoses using the gas chromatographic method of Sweeley *et al.* (1963). Twelve peaks were detected and five were identified. The unidentified products were attributed to degradation during hydrolysis of the methylated xylan. No effort was made to determine the relative composition of the xylan from these data, nor were the linkages between the components identified.

MATERIALS AND METHODS

Test Organism

The source of glycogen for this study was the boll weevil, Anthonomus grandis Boheman.

Rearing

Cultures

Boll weevils were obtained either from cotton fields in the Southeastern United States or from Mexico. They were maintained in the laboratory for several years and were identified by the locality in which they were collected. The major part of the glycogen used in this study was obtained from the Castleberry culture (Castleberry, Alabama) with the remainder extracted from the Dunlap strain (Tallulah, La.) or the Mexico strain. Mexico weevils were obtained from the Boll Weevil Research Laboratory, State College, Mississippi.

Maintenance of Parent Stocks

Eggs from each culture were set aside every two or three months to insure a continuous supply of weevils. Upon emergence, adult weevils were placed in one gallon ice cream cartons, fitted with screen tops, and fed either squares (cotton flower buds) or bolls daily. These squares and bolls were collected and dissected for eggs; from these eggs the test organisms were obtained.

Egg Extraction

Eggs were extracted from the squares and bolls by the procedure of Betz (1965). This procedure employed mechanical methods of separating cotton square debris from the eggs without injuring the eggs. The squares were chopped in a household food chopper in about 250 ml water. The agitating action of the chopper blades separated parts of squares from eggs. The eggs then settled to the bottom of the jar where they were protected by a Teflon disc placed on the convex bottom of the jar.

The egg-water suspension was either washed through a series of sieves (U. S. Bureau of Standards Nos. 8, 20, 30, and 60) and rinsed off the No. 60 sieve into a small beaker or transferred (after chopping) to a large beaker. The water in the beaker was swirled gently; this movement drew the eggs to the vortex, where they were removed to a smaller beaker with an inverted five ml volumetric pipette fitted with a rubber bulb. The eggs were surface sterilized in the small beaker. However, prior to sterilization, as much water as possible was removed with the pipette so as not to dilute the initial sterilizing solution.

Egg Sterilization

Eggs were surface sterilized (Nettles and Betz, 1965) by the following procedure:

1. Eggs were allowed to stand in aqueous 18% cupric sulfate solution for 3-5 min. and gently swirled several times for good surface contact.

2. Cupric sulfate was drawn from the bottom of the beaker with a pipette resting on the bottom (eggs float and do not interfere with the removal of cupric sulfate solution). The eggs were then rinsed with sterile water and the water carefully removed.
3. A solution of mecuric chloride (0.04%) in 25% ethanol was added and was in contact with the eggs for 3-5 min.
4. The eggs were rinsed with sterile water and poured into a sterile petri dish for implanting.

Egg Implanting and Maturation

The surface sterilized eggs were implanted onto larval diet by the capillary tube method of Betz (1965). Immediately after sterilization, the eggs were transferred to a sterile petri dish and submerged in sterile water. A Pasteur capillary pipette, fitted with a small rubber bulb, was used to transfer the eggs from the holding dish. The larval media were scratched with a device made by taping three biological probes together. The device was flame sterilized before each dish was scratched.

The egg-water suspension was drawn into the capillary pipette and ejected uniformly over the diet. Usually two transfers were necessary to place the optimal number of eggs (50-70) onto the media. Excess water (added with the eggs) was removed by tilting the petri dish and allowing the water to drain over the lower side. Eggs adhered to the roughened diet surface and were not removed when the excess water was drained out.

To decrease the chance of microbiological contamination, the implanted dishes were opened and inverted on a sterile surface for 1-8 hours.

The implanted dishes were removed to a 30°C holding room for the maturation period of usually 14-17 days. Since Earle and Newsom (1964) showed that boll weevil diapause is at a minimum at a long day, the photoperiod for both larvae and adults was 13 hours of light and 11 hours of darkness. Therefore, maintenance of a 13 hour photophase was conducive to oviposition.

Larval Diet.

The larval diet, essentially that described by Earle, et al. (1959), was prepared in 1600 gram batches and consisted of:

Distilled water	1600 ml
Agar	48 g
Sucrose	32 g
Soy protein	48 g
Wesson salts	4.8g
Cholesterol	0.8g
Cystine HCL	0.4g
Choline chloride	1.6g
Acetone powder of cotton squares	80 g
Yeast extract	16 g
Tegosept	1.6g
Sorbic acid	1.6g
B-Vitamins	52 mg
Inositol	320 mg

Approximately 24 g of hot medium (Diet B) were poured into each sterile plastic petri dish (100 x 15 mm). The diet was stored at room temperature for periods up to two months before becoming unsatisfactory for use.

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Egg Glycogen

Egg glycogen was c.w.e. from Mexico eggs stored under two holding conditions and were obtained from Dr. R. Gast (Boll Weevil Research Laboratory, State College, Mississippi). The first extraction was made with eggs stored under methanol at -70°C for two months and the second with eggs stored in ethanol at -70°C for less than two months. Each extraction was carried out with duplicate 10 ml volumes of eggs after the removal of the excess methanol or ethanol.

Larval Glycogen

Glycogen from late last instar larvae was obtained by collecting and extracting 50 mg of weevils with either 5% Trichloroacetic acid (TCA) or cold chloroform-glycine buffer. The TCA extraction was performed on 1600 stored larvae (at -20°C in ethanol) but the cold water extraction (c.w.e.) was carried out with unstored larvae. These larvae were removed from the diet and immediately dropped into the cold chloroform-glycine mixture (0°C).

Adult Glycogen

Samples of adult glycogen were extracted from 14-day old reproducing weevils. After removal from the rearing media, the weevils were placed in one gallon ice cream cartons and fed bolls or squares daily for 14 days. They were removed from these cartons, sexed, weighed and stored in ethanol (95%) at -20°C. They remained stored

until homogenization at a later date. Combinations of groups (about 50 weevils per group) were selected for extraction with either TCA or cold chloroform-glycine.

Glycogen Standard

The primary glycogen standard was "Mann Assayed" rabbit liver glycogen (Mann Research Laboratories, Inc., New York, New York). This glycogen was extracted by the procedure of Somogyi (1934) which consists of prolonged exposure in hot alkali (KOH at 100°C). This sample was the purest available commercial glycogen and was obtained from the livers of fattened rabbits.

GLYCOGEN EXTRACTION AND PURIFICATION

Extraction with 5% Trichloroacetic Acid

One gram of boll weevil larval glycogen was extracted from 1600 larvae that had been stored at -20°C in ethanol for one to five weeks. The larvae were divided into four groups of 400 each and were homogenized with 30 volumes of 80% ethanol. Homogenization was carried out at high speed (45,000 rpm) with a VirTis "45" homogenizer. The homogenate was allowed to stand overnight at room temperature to allow the glycogen to precipitate from the ethanol extract. After mixing well, the extract was centrifuged at 12,000 x g for 10 minutes at 20°C.

The ethanol precipitate was rehomogenized twice with 30 ml of 5% trichloroacetic acid and centrifuged at 12,000 x g for 10 minutes. The supernatants were combined and diluted with 95% ethanol to give a final concentration of 80%. After standing overnight at room temperature, the TCA-ethanol mixture was centrifuged at 12,000 x g for 10 minutes. The precipitated glycogen was dissolved in water, stirred well, and reprecipitated by the addition of five volumes of 95% ethanol. After centrifuging, the precipitated glycogen was re-extracted with water-ethanol (1:5/v:v). Since the purified glycogen (in some cases) did not precipitate from the last

ethanol extraction, it was necessary to remove the water-ethanol solution under reduced pressure with a rotary evaporator. The crystalline glycogen was dried in a vacuum desiccator over calcium chloride.

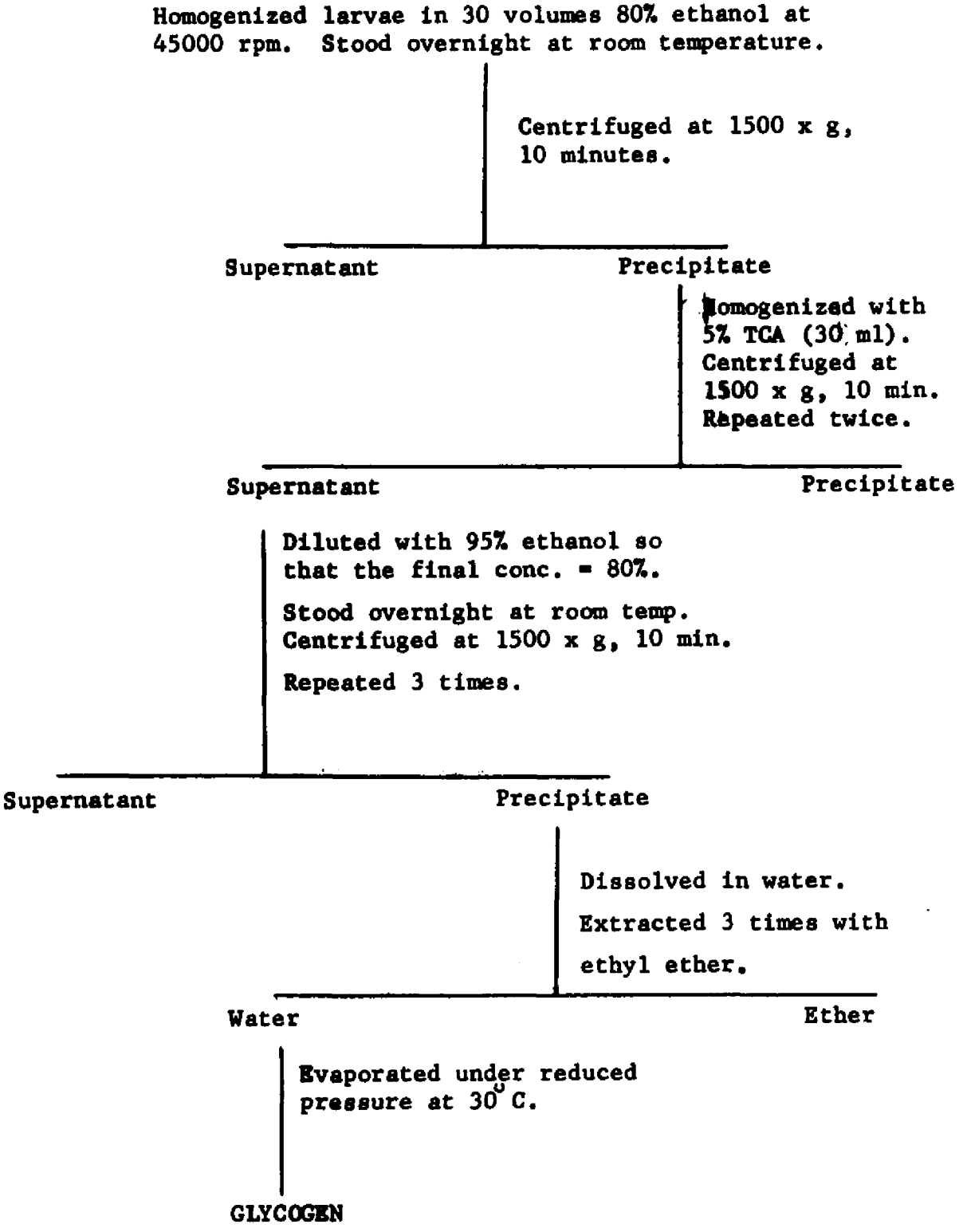
The powder was then dissolved in water and extracted three times with anhydrous ethyl ether. The water solution was separated and dried under reduced pressure at 30°C. The glycogen was a fine, crystalline powder. A schematic representation of the extraction procedure is presented in Figure 1.

Extraction with Cold Chloroform-Glycine Buffer

The initial homogenization of the cold water method (c.w.e.) was carried out at high speed in chloroform-glycine buffer; four volumes glycine buffer (0.2M;pH 10.5) and two volumes water-washed chloroform were used at 2 to 4°C, according to the method of Bueding and Orrell (1965). Enzymic alterations were prevented by the chloroform while glycine prevented decreases in pH accompanying the extraction of glycogen from biological material. After centrifuging the initial homogenate (180 x g; 10 minutes, 2-4°C), the glycine layer, removed by aspiration, was frozen. The chloroform layer and interphase were rehomogenized (2-4°C) with two volumes of glycine buffer. After centrifuging at 180 x g for 10 minutes, the supernatant layer was combined with the initial glycine layer and frozen. This rehomogenization was repeated four times.

The thawed glycine layers were centrifuged in a preparative Spinco Model L refrigerated centrifuge at 100,000 x g for four hours

Figure 1. Extraction of Boll Weevil Larval Glycogen with 5% Trichloroacetic Acid

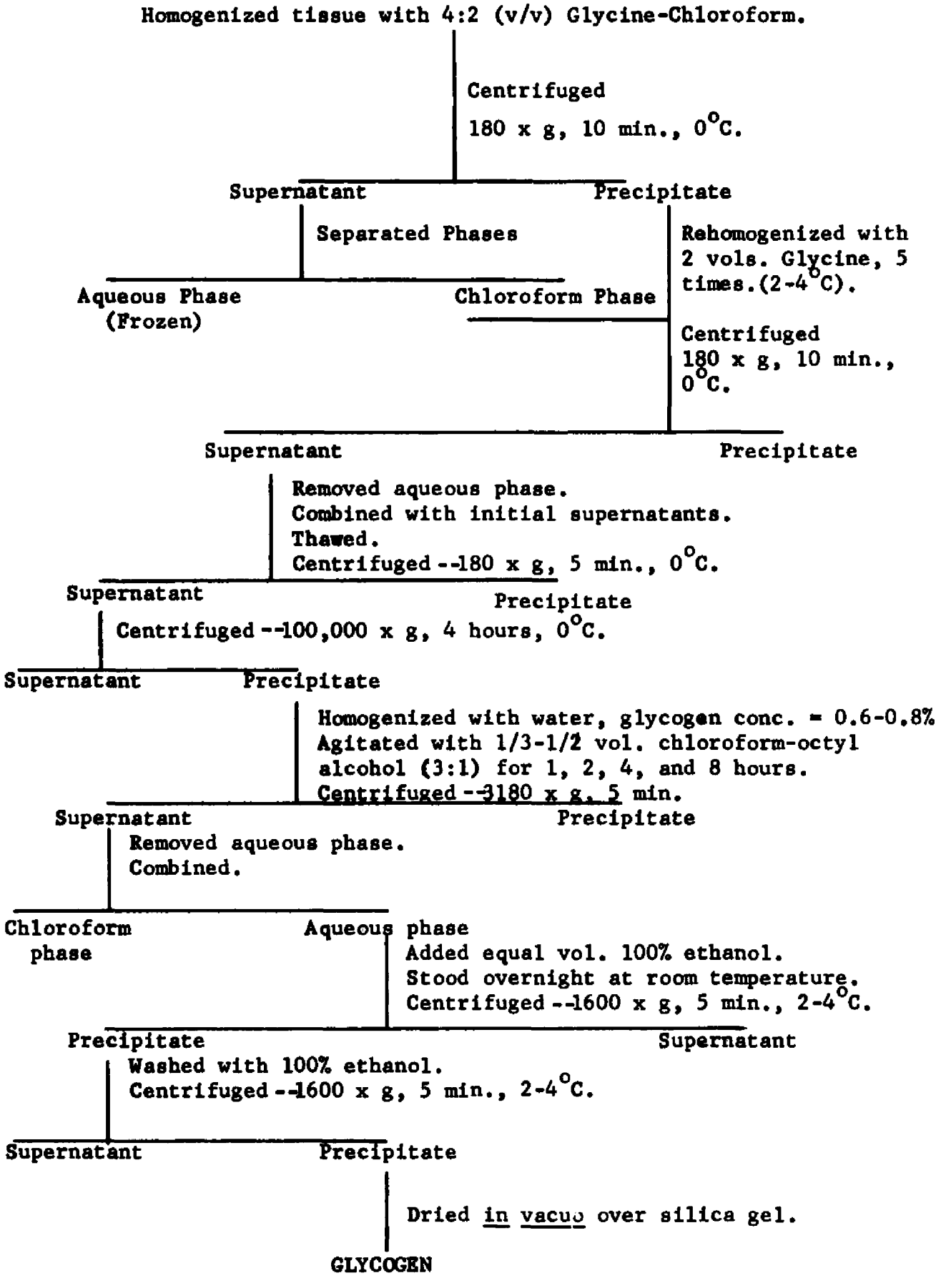

Homogenized larvae in 30 volumes 80% ethanol at 45000 rpm. Stood overnight at room temperature.
Centrifuged at 1500 x g, 10 minutes.
Supernatant
Precipitate
Homogenized with 5% TCA (30 ml). Centrifuged at 1500 x g, 10 min. Repeated twice.
Supernatant
Precipitate
Diluted with 95% ethanol so that the final conc. = 80%.
Stood overnight at room temp. Centrifuged at 1500 x g, 10 min.
Repeated 3 times.
Supernatant
Precipitate
Dissolved in water.
Extracted 3 times with ethyl ether.
Water
Ether
Evaporated under reduced pressure at 30° C.
GLYCOGEN

at 0°C. The gelatinous precipitate (containing the glycogen) was dissolved in water so that the glycogen content did not exceed 0.6%. The glycogen solution was agitated for three to five minutes with 1/3 its volume of a mixture of chloroform:n-octyl alcohol (3:1) according to Sevag (1934). The mixture was centrifuged at 9200 x g for 10 minutes and the aqueous supernatant was carefully transferred to centrifuge tubes. Care was taken not to disturb the interphase during aspiration of the aqueous phase. This treatment was repeated for progressively longer periods (1, 2, 4, and 8 hours) with careful removal of the aqueous phase after each treatment.

Glycogen was isolated from the combined aqueous phases by the addition of 100% ethanol (1:1; v/v), allowing to stand overnight at room temperature, and subsequent centrifuging at 1600 x g for 10 minutes at 2-4°C. After washing with 100% ethanol and centrifuging, the c.w.e. glycogen was dried *in vacuo* over silica gel. Figure 2 shows the schematic separation and isolation of glycogen from boll weevil homogenates.

Cold water extracted glycogen was obtained from eggs, larvae, and adults, as well as larvae reared on a high sucrose diet. The following designations were assigned to the glycogen samples used in this study:

Figure 2. Extraction of Boll Weevil Glycogen with Cold Chloroform-Glycine Buffer.


Homogenized tissue with 4:2 (v/v) Glycine-Chloroform.
Centrifuged
180 x g, 10 min., 0°C.
Supernatant
Precipitate
Separated Phases
Rehomogenized with 2 vols. Glycine, 5 times.(2-4°C).
Aqueous Phase (Frozen)
Chloroform Phase
Centrifuged
180 x g, 10 min., 0°C.
Supernatant
Precipitate
Removed aqueous phase.
Combined with initial supernatants.
Thawed.
Centrifuged--180 x g, 5 min., 0°C.
Supernatant
Precipitate
Centrifuged--100,000 x g, 4 hours, 0°C.
Supernatant
Precipitate
Homogenized with water, glycogen conc. = 0.6-0.8%
Agitated with 1/3-1/2 vol. chloroform-octyl alcohol (3:1) for 1, 2, 4, and 8 hours.
Centrifuged--3180 x g, 5 min.
Supernatant
Precipitate
Removed aqueous phase.
Combined.
Chloroform phase
Aqueous phase
Added equal vol. 100% ethanol.
Stood overnight at room temperature.
Centrifuged--1600 x g, 5 min., 2-4°C.
Precipitate
Supernatant
Washed with 100% ethanol.
Centrifuged--1600 x g, 5 min., 2-4°C.
Supernatant
Precipitate
Dried in vacuo over silica gel.
GLYCOGEN

Sample Number	Extraction	Source
103	c.w.e.	Castleberry larvae, Diet B
109	c.w.e.	Castleberry adults, Boll-fed, stored 12 months in ethanol at -20°C
112	c.w.e.	Mexico eggs, stored in methanol two months at -70°C
120	c.w.e.	Castleberry larvae, Diet B, plus 20% sucrose
130	TCA	Castleberry larvae, Diet B, plus 20% sucrose
133	c.w.e.	Mexico eggs, stored in ethanol two months at -70°C
RL	KOH	Rabbit Liver, Mann Research Biochemicals

PHYSICAL ANALYSES

Carbohydrate Content

The carbohydrate content of the standard and boll weevil glycogens was determined by the anthrone procedure of Carroll et al. (1956). The anthrone reagent, prepared fresh weekly, consisted of 0.05% anthrone and 1% thiourea dissolved in 72% sulfuric acid. This mixture was heated to 80°C and, after cooling to room temperature, was stored in the refrigerator at 5°C.

Glycogen solutions were prepared so that a 100 µl aliquot contained approximately 100 µg of glycogen. After the addition of 0.9 ml of water, 5 ml of the anthrone reagent were added while the tubes were submerged in ice water. Each tube was thoroughly mixed with a Vortex, Jr. tube stirrer and capped with small condensers prepared from four inch pieces of pyrex tubing and rubber stoppers. The tubes were then submerged for exactly 15 minutes in a boiling water bath maintained at 100°C. The tubes were returned to the ice water bath and agitated. After about five minutes, the glycogen content of the samples was determined at 620 mµ with a Beckman DB Spectrophotometer. Glucose was used as the standard and the glucose content of each tube was converted to glycogen by the following relationship:

$$\frac{DU}{DS} \times 0.1 \times \text{aliquot} \times 0.9 = \text{mg glycogen}$$

where

DU = Optical Density of the Sample (Unknown)

DS = Optical Density of the Standard

0.1= Glucose concentration of the Standard (in mg)

0.9= Factor for Converting Glucose to Glycogen

Protein Content

The protein content of the TCA extracted glycogen was determined by the methods of Moore and Stein (1954) and of Koch and McMeekin (1924). Triplicate 1 mg samples were hydrolyzed with 6N HCL for 18 hours at 100°C. The acid hydrolyzate was evaporated to dryness in a desiccator over NaOH. The residue was dissolved in water twice and re-dried. Triple distilled water was used in these analyses.

The samples (approximately 0.1 μM glycogen) were heated in a tube heater at 100°C for 15 minutes with ninhydrin solution. After diluting with 50% ethanol and mixing well on a mechanical shaker, the tubes were cooled to 30°C. The readings were made with a Bausch and Lomb Spectronic 20 at 570 mμ.

The Koch and McMeekin method was carried out as follows: A 5 ml sample of boll weevil glycogen (in triplicate) was placed into a 200 x 25 mm pyrex test tube, 1 ml of 1:1 sulfuric acid added along with a small quartz pebble, and heated over a microburner to evaporate off the water. When charring began, the tube was raised

so that the tip of the flame just touched the bottom of the tube and heating was continued until no further darkening occurred. After removing the flame, the tube was allowed to cool and 1 drop of 30% hydrogen peroxide was dropped directly into the tube. The tube was returned to the flame and heated to boiling. If the solution did not decolorize, the hydrogen peroxide addition was repeated. Finally, the solution was boiled gently for about five minutes. The solution was then cooled, transferred to a 50 ml volumetric flask, and about 35 ml of water were added.

When the standards and the blanks were ready, 12 ml of modified Nessler reagent were added to all flasks, swirling the contents to promote quick and uniform mixing. The contents of the flasks were immediately diluted to the 50 ml mark with water, stoppered, and mixed. All samples were allowed to stand for 10 minutes before they were read in a Bausch and Lomb Spectronic 20.

Since the c.w.e. glycogens were 95-98% carbohydrate material, protein, moisture, and ash were not determined on these samples.

Moisture Content

The moisture content of the TCA extracted larval glycogen was determined by daily weighings of 50 mg samples stored *in vacuo* over phosphorus pentoxide. Duplicate samples of boll weevil and standard glycogen were weighed into tared glass vials and moisture determined by the loss after reaching constant weight.

Ash Content

The ash content of TCA extracted glycogen was determined with a Muffle furnace held at 600°C for 12 hours. Duplicate 50 mg samples of boll weevil and standard glycogen were weighed into tared platinum crucibles and placed into the Muffle furnace. One hour was taken to slowly raise the temperature to its maximum of 600°C.

Additional Analyses

The combined investigations on the purity of the TCA extracted boll weevil glycogen did not account for 100% of the sample. Additional work was carried out in an attempt to determine the composition of the remainder of this sample.

Duplicate 5 mg samples of standard and boll weevil glycogen were chromatographed through a column of Sephadex G-200 gel. A bed volume of 120 ml was used and 24, 10 ml fractions were collected with an automatic fraction collector. A 1 ml aliquot of each tube was subjected to the anthrone test and total carbohydrate determined with glucose as the standard.

Paper electrophoresis of the borate complexes of 1% solutions of standard and boll weevil glycogen was carried out at high voltage (500v) and low voltage (300v), according to the method of Foster *et al.* (1956). Borate buffer (pH 10.0) was prepared by mixing 0.74% boric acid and 0.4% sodium hydroxide solutions. The paper electrophoresis strips were subjected either to high voltage for four hours or to low voltage for eight days in a constant temperature room at 5°C. The strips were developed with 0.4% iodine in ethanol.

Optical Rotation

The optical rotation of TCA extracted larval glycogen was determined with a Rudolph Polarimeter (Model 70) in an aqueous solution at 20°C using the D-line of sodium. The $[\alpha]_D^{20}$ was calculated from the following relationship:

$$[\alpha]_D^{20} = \frac{100 \times \alpha_D^{20}}{c \times 1}$$

where $[\alpha]_D^{20}$ is the specific rotation at 20°C measured with the D-line of sodium, α_D^{20} is the observed rotation, c the concentration expressed in grams per 100 ml of solution, and 1 the length of the polarimeter tube in decimeters.

Iodine Binding Power

The iodine absorption power of the boll weevil glycogen samples was determined using the method of Archibald et al. (1961). The analyses were carried out at room temperature; solutions were prepared with 0.01% glycogen in 0.02% iodine dissolved in 0.2% potassium iodide solution. After standing for 10 minutes, absorption was obtained with a Beckman DU and a recording DB spectrophotometer.

Infra-red Determinations

Infra-red absorption of solid glycogen samples was determined with a Beckman IR-5 spectrophotometer. Solid samples of 0.5 to 1.0 mg were made into a pellet with potassium bromide and scanned with the recording Beckman IR-5. Identification of the IR "fingerprint" was

based on that obtained with the standard glycogen sample and by calculation of the Sadtler Index.

Molecular Weight Determinations

Molecular weights of glycogen were obtained by the method of Orrell and Bueding (1965) and were performed at the Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland. The determinations were carried out in a Spinco Model E analytical ultracentrifuge at approximately 9300 rpm. The exact speeds were measured with a photo-electric digital tachometer. A 1% solution of the glycogen sample in water was used at 20°C with a phase plate angle of 45°. Analyses were made using the Schlieren optical system and an acceleration of 1 minute 10 seconds linear rate. Plates were measured on a Gaertner 2-axis micrometer-comparator and the sedimentation curves were corrected to zero concentration by the method of Baldwin (1954). Since actual computations were quite involved, an IBM computer was programmed to correct for effects of concentration, temperature, viscosity, etc. As a result complete normalized sedimentation curves, fully corrected to finite concentration, were obtained. The sedimentation patterns so obtained showed relative concentrations on the ordinate scales (RCS-i.e. as if they had the same total concentration) and the abscissa values were in svedberg units (1 svedberg = 1×10^{-13} cm/dyne sec.). These S-values were also corrected values at zero concentration and 20°C in water. Therefore, all sedimentation curves were comparable.

CHEMICAL ANALYSES

Monosaccharide Released on Complete Acid Hydrolysis

Ten samples of TCA larval glycogen and three samples of c.w.e. egg and larval glycogen were subjected to vigorous acid hydrolysis in 1N sulfuric acid. To each sample dissolved in 1 ml water was added 1 ml of 2N sulfuric acid, heated for 2.5 hours at 100°C, and cooled. The hydrolyzates were diluted to approximately 50 ml with water and neutralized (while controlling the pH) with barium hydroxide. The precipitated barium sulfate was removed by centrifugation, washed once with water, and reacted with anthrone reagent to determine the amount of carbohydrate material trapped by the precipitate. Ethanol was added to an aliquot of the supernatant to precipitate any unhydrolyzed glycogen.

The remaining supernatant was evaporated to dryness under reduced pressure at 30°C. Six of the 10 TCA samples and all of the c.w.e. samples were dissolved in water for thin-layer chromatography and glucose oxidase assay. Three of the remaining TCA samples were dissolved in pyridine, reacted with trimethylchlorosilane and hexamethyldisilazane, and subjected to gas-liquid chromatography according to the method of Sweeley *et al.* (1963). The three c.w.e. glycogen samples were evaporated to dryness following thin-layer and glucose oxidase analyses, 1 ml pyridine added, and reacted with the silanization reactants for gas-liquid chromatography.

Thin-layer chromatography was carried out on three TCA hydrolyzates and all c.w.e. hydrolyzates using three solvent systems that were found to be most suitable for the separation of hexoses. The monosaccharides were determined by the R_f and the color obtained with the spray reagent.

Enzymic determinations were carried out using three of the TCA hydrolyzates and all the c.w.e. hydrolyzates by the method suggested by the manufacturer of the enzyme (Worthington Biochemical Corp.). Aliquots of the hydrolyzates were incubated with glucose oxidase at room temperature for exactly 10 minutes and the resulting color read in a Beckman DB Spectrophotometer at 400 mμ. The glucose oxidase was prepared immediately before use and the reaction was terminated by the addition of 4M hydrochloric acid. At least five minutes were allowed for the color intensity to stabilize prior to reading in the DB.

Gas chromatographic analyses were carried out on three of the TCA hydrolyzates and all the c.w.e. hydrolyzates using a Micro Tex Pesticide Residue Analyzer (Model 1607), modified with a single hydrogen flame detector. Retention data were obtained using authentic α-glucose, β-glucose, dextrose, and mutarotated α- and β-glucose.

The remaining TCA hydrolyzate was subjected to thin-layer chromatography, glucose oxidase activity, and gas-liquid chromatography, in the same manner as the c.w.e. glycogen hydrolyzates.

β-Amylolysis Limit Dextrin

The β-Amylolysis Limit Dextrin (L.D.) was determined for the several boll weevil glycogen samples as well as the standard using a modified method of Barker et al. (1950). Incubation tubes, micro-Millipore filters, and syringes were autoclaved immediately before use. The purified enzyme (obtained in saturated ammonium sulfate from Worthington Biochemical Corp.) was thoroughly mixed with acetate buffer (pH 4.7) and a suitable aliquot was introduced into the tubes held at 20°C. Three times recrystallized β-amylase, prepared by the method of Balls et al. (1948), was used and introduced into the tubes by passing the solution through a micro-Millipore filter. After stabilizing the temperature of the enzyme-buffer mixture, the glycogen solutions were prepared and injected into the tubes using sterile techniques. After incubating for exactly four hours at 20°C, each tube was injected with five ml 95% ethanol. The tubes were removed from the water bath, stirred well, and placed at -20°C. Samples were removed singularly, evaporated under reduced pressure at 30°C, and dissolved in 2 ml water. A small aliquot (usually 1-3 μl) was removed and subjected to thin-layer chromatography for detection of the maltose released and the L.D. The remainder of the sample was placed on a column of Sephadex G-200 and eluted with distilled water. Two ml fractions were collected with a fraction collector.

One ml aliquots of each tube were removed and analyzed by the anthrone procedure of Carroll et al. (1956). The per cent

maltose released was determined from the per cent of anthrone positive material in the second series of fractions as compared to the total anthrone positive material of the eluant. The eluant was distinctly divided into two regions of reducing material; that given by the L.D. in the early fractions and that given by the maltose in the latter fractions.

Maltose Determination

Maltose obtained by the β-amylase digestions was determined qualitatively by thin-layer and gas-liquid chromatography. The tubes containing the maltose fractions were taken to dryness under reduced pressure at 30°C and were combined. The combined residue was taken up with 1 ml anhydrous pyridine and a suitable aliquot was spotted on Silica Gel H plates for thin-layer chromatography in two solvent systems. Another aliquot was spotted, developed, and reacted with spray reagent for "rough" quantitation with the recording densitometer. A standard curve was obtained for standard maltose with the densitometer.

Gas-liquid chromatography was carried out on the octa-trimethylsilyl derivative of maltose. An aliquot of the pyridine solution was treated with 0.1 ml hexamethyldisilazane and 0.05 ml trimethylchlorosilane, and gas-liquid chromatography of the derivative was accomplished at high temperature on the non-polar liquid phase, SE-30.

The remaining pyridine fraction was lyophilized and stored in the freezer. Replicate determinations from the β-amylase digestion were pooled for later investigation of their linkages.

Phosphorylase Limit Dextrin

All boll weevil glycogens were degraded by incubation with phosphorylase b according to the method of Bueding and Hawkins (1964). Approximately 0.08 to 0.1 mg of each glycogen sample was incubated for two hours at 30°C in a total volume of 0.26 ml containing 0.3 mole of adenosine monophosphate (AMP), 0.1 ml of phosphorylase b solution, and 200 μmoles of potassium phosphate buffer (pH 7.0). Eight times recrystallized phosphorylase b was prepared according to the method of Fisher and Krebs (1958). The glucose-1-phosphate produced by the action of the phosphorylase was converted to glucose-6-phosphate in the presence of phosphoglucomutase and was analyzed spectrophotometrically in the presence of nicotinamide adenine dinucleotide phosphate (NADP), catalytic amounts of glucose-1,6-diphosphate, and glucose-6-phosphate dehydrogenase. The glucose-1-phosphate content of the samples was calculated on the basis of the molar extinction coefficient of $NADPH_2$ at 340 mμ. The L.D. from phosphorylase was determined by the difference obtained from the glucose-1-phosphate yielded and the amount of starting material.

Average Chain Length

The average chain length of standard and boll weevil glycogen was determined both enzymically and chemically. The enzymatic method of Bueding and Hawkins (1964) allowed the simultaneous determinations of the phosphorylase limit dextrin and the average chain length. The average chain length (CL) was calculated from data obtained during

the complete degradation of glycogen (or the phosphorylase limit dextrin) by the combined action of phosphorylase b and amylo-1,6-glucosidase.

An aliquot of the glycogen solution prepared for degradation by phosphorylase b alone was incubated with excess phosphorylase b in the presence of amylo-1,6-glucosidase (containing transglucosylase activity). Free glucose was produced by the action of the glucosidase at the branch points and, as previously discussed, glucose-1-phosphate by the action of phosphorylase b. Therefore, after complete degradation by this enzymatic system, the ratio of free glucose to glucose-1-phosphate was a measure of the branch points (α1,6) in the glycogen samples.

Free glucose produced by this system was determined using hexokinase, adenosine triphosphate (ATP), and glucose-6-phosphate dehydrogenase by means of a modification of Bueding et al. (1961) of the procedure of Slein et al. (1950). The glucose-1-phosphate resulting from this enzymic conversion was determined using the system described for the glucose-1-phosphate produced in the phosphorylase limit dextrin analysis. The sequence of reactions is as follows:

$$\text{glucose} \xrightarrow[\text{6-phosphate dehydrogenase}]{\text{Hexokinase, ATP, Glucose-}} \text{glucose-1-phosphate}$$

$$\text{glucose-1-phosphate} \xrightarrow{\text{Phosphoglucomutase}} \text{glucose-6-phosphate}$$

$$\text{glucose-6-phosphate} + \text{NADP} \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-Phosphate}} \text{6-phosphogluconate} + \text{NADPH}_2$$

After corrections were made for changes in volume, the glucose-1-phosphate content of the samples was calculated on the basis of the molar extinction coefficient of $NADPH_2$ (6220). Glucose was determined by the difference in the glucose-1-phosphate content of the glycogen sample subjected to total degradation (with both enzymes) and the glucose-1-phosphate content of the sample digested with phosphorylase b alone.

The average chain length of the samples was calculated from the ratio of glucose to glucose-1-phosphate produced by this system. The amount of free glucose present was indicative of the per cent branch points and the average chain length was based on total glucose in the chains and the per cent branch points.

The average chain length of these samples was also determined by a non-enzymic method, i.e., gas-liquid chromatography of the hydrolytic products after exhaustive methylation, according to the method of Betz, Nettles and Novak (unpublished data).

Glycogen samples were methylated by a modification of the procedure of Kuhn et al. (1960). They have suggested reaction times sufficient for the methylation of disaccharides, so it was necessary to increase the reaction time to obtain complete methylation of the glycogen. Methylation was carried out in dimethylformamide and methyl iodide at 20°C while adding, from time to time, a stoichiometric amount of silver oxide. The mixture was stirred vigorously for about 15 minutes after which the temperature gradually rose to 30°C. The complete methylation took about 12-18 hours at this temperature; however, glycogen required five days.

This mixture was removed from the water bath and centrifuged for 10 minutes at room temperature. The supernatant was carefully decanted and the precipitate washed with dimethylformamide 4 - 5 times. After combining these washings with the initial supernatant, the precipitate was washed 4 - 5 times with chloroform. These washings were combined with the earlier supernatants and were mixed with 1% potassium cyanide solution in a separatory funnel. The combined solutions were extracted with chloroform 4 - 5 times (1 vol. chloroform for each 5 vols. KCN solution). The chloroform extract was washed with an equal volume of water 3 - 4 times and dried over sodium sulfate.

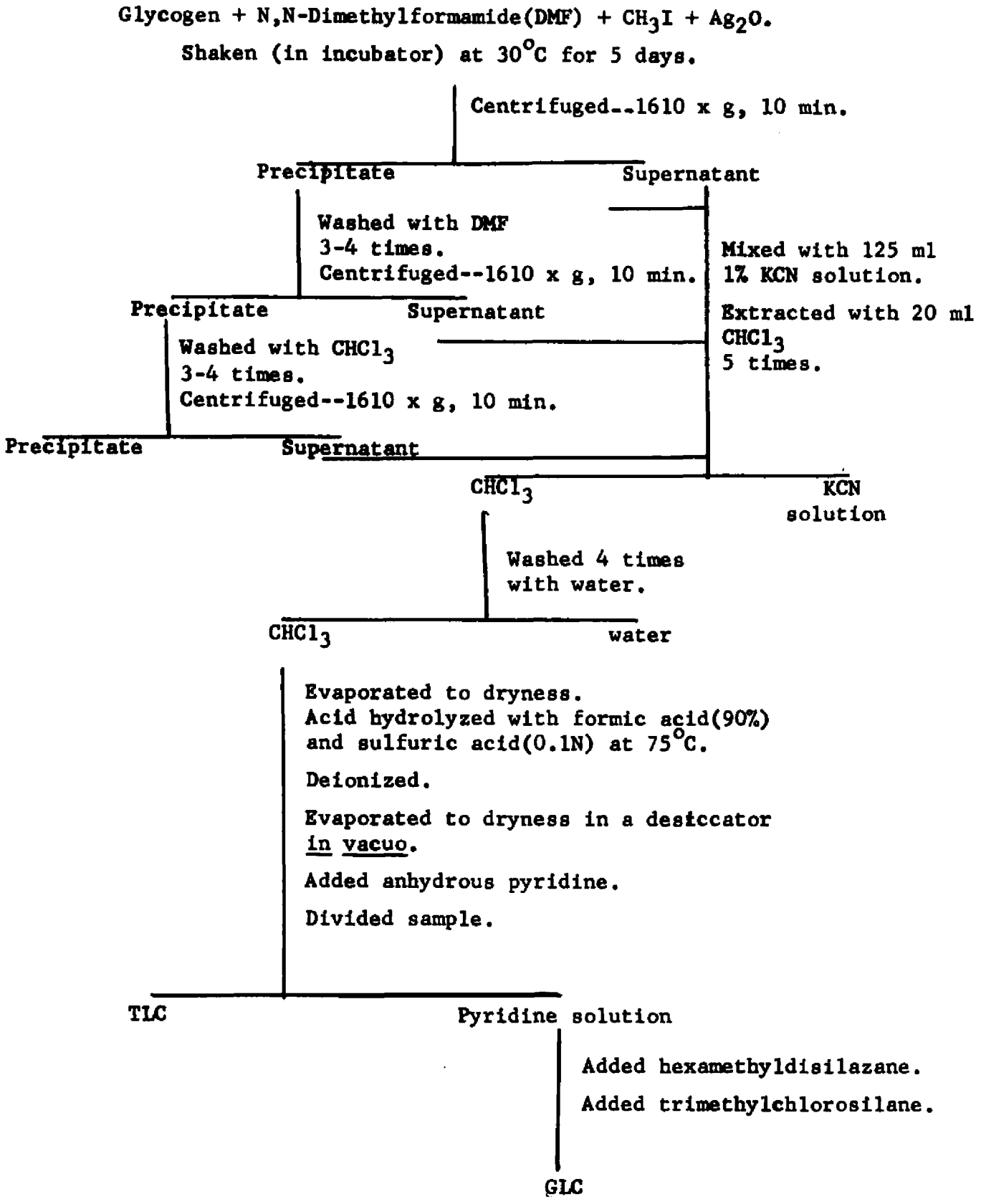
The chloroform solution (containing the methylated glycogen) was evaporated at reduced pressure and 30°C to a pale yellow liquid. After a comprehensive study was carried out to assess the relative amounts of demethylation and degradation during different acid hydrolysis conditions, the pale yellow liquid was evaporated overnight in a vacuum desiccator. The methylated glycogen was digested with 90% formic acid at 75°C for two hours, according to a modification of the method of Bouveng and Lindberg (1965). After cooling, an equal volume of 0.1N sulfuric acid was added and the mixture was held overnight at 75°C in a tube heater. The released methyl glucopyranoses were diluted with water and passed through either a layered bed of Dowex 1 and Dowex 50 ion exchange resins or Amberlite IR-4B anion exchange resin. A bed volume of 100 ml was used and 25-5ml fractions were collected with an automatic fraction

collector for the layered Dowex resins. Ten 15 ml fractions were eluted from the Amberlite resin using a bed volume of 40 ml. A schematic representation of the methylation, hydrolysis, and deionization is presented in Figure 3.

To determine the per cent recovery of these methylated glucopyranoses off the ion-exchange resins, a known amount of methyl-α-D-glucoside was dissolved in one ml of anhydrous pyridine. One-half of the sample was placed on the resin column and eluted with a sufficient volume of water. The remaining sample was silanized directly and an aliquot gas-liquid chromatographed. The eluate from the resins was evaporated to dryness under reduced pressure at 30°C. The residue was dissolved in 0.5 ml pyridine, transferred to a small opticlear vial, and silanized. An aliquot of this solution was gas-liquid chromatographed under the exact conditions as the un-deionized sample. The per cent recovery was based on the amount of methyl-α-D-glucoside eluted from the resins.

The fractions which had been determined to contain the methylated sugars, were taken to dryness under reduced pressure at 30°C, and initially each residue was dissolved in 0.5 ml pyridine and chromatographed by thin-layer techniques. Later all fractions were collected and evaporated together and the final residue was dissolved in one ml anhydrous pyridine. An aliquot of this solution was removed and subjected to thin-layer chromatography in n-butanol: ethanol:water:ammonia (40:10:49:1), according to the method of Hirst et al. (1949, paper chromatographic method adapted to thin-layer methods). The thin-layer chromatography was carried out on Silica

Figure 3. Methylation, Hydrolysis, and Deionization of Boll Weevil Glycogen.


Glycogen + N,N-Dimethylformamide(DMF) + CH_3I + Ag_2O.
Shaken (in incubator) at 30°C for 5 days.
Centrifuged--1610 x g, 10 min.
Precipitate
Supernatant
Washed with DMF
3-4 times.
Centrifuged--1610 x g, 10 min.
Mixed with 125 ml
1% KCN solution.
Precipitate
Supernatant
Extracted with 20 ml
$CHCl_3$
5 times.
Washed with $CHCl_3$
3-4 times.
Centrifuged--1610 x g, 10 min.
Precipitate
Supernatant
$CHCl_3$
KCN
solution
Washed 4 times
with water.
$CHCl_3$
water
Evaporated to dryness.
Acid hydrolyzed with formic acid(90%)
and sulfuric acid(0.1N) at 75°C.
Deionized.
Evaporated to dryness in a desiccator
in vacuo.
Added anhydrous pyridine.
Divided sample.
TLC
Pyridine solution
Added hexamethyldisilazane.
Added trimethylchlorosilane.
GLC

Gel G, prepared in a water solution, and the plates were sprayed with either 1% p-anisidine in 10 ml methanol diluted to 100 ml with n-butanol or with a 5% aqueous solution of sulfuric acid. The plates were developed at 110°C for 20 minutes.

Frequently the chromatography was carried out on small plates (5 cm x 20 cm) so that they could be run through a recording densitometer, modified to scan such plates. In this way it was possible to record the relative amounts of tetra:tri:dimethylglucopyranoses, their ratio (% composition) and complementary data for that obtained by gas-liquid chromatography.

After aliquots were taken for thin-layer chromatography, the remaining pyridine solutions were transferred to small glass vials, silanized by the method of Sweeley et al. (1963), and subjected to gas-liquid chromatography.

Exterior Chain Length

The average exterior chain length was determined enzymically by degradation with phosphorylase b and β-amylase. This was accomplished by incubating the glycogen solutions for phosphorylase limit dextrin determination with an excess of phosphorylase b. The glucose-1-phosphate released by the exterior chains was determined enzymically by the method of Bueding and Hawkins (1963) previously described. The average exterior chain length was calculated from the per cent of phosphorylase b cleavage and the limit of phosphorylase action on glycogen.

The average exterior chain length was also determined by digestion of the glycogens (of known chain length) with crystalline β-amylase. The per cent β-amylolysis, in the acetate buffer system previously described, was used to calculate the exterior chain length from the relationship presented by Manners and Wright (1962), which was as follows:

ECL = No. glucose residues removed by β-amylase + 2.5

Interior Chain Length

The interior chain length (ICL) at the present time can be calculated only from information obtained for the average exterior and chain lengths. Manners (1962) stated that the average chain length minus the exterior chain length -1 is an approximation of the average interior chain length. According to Bueding (personal communication), the average interior chain length is determined from the relationship CL=ECL+ICL, when the average chain length is obtained enzymically. The latter relationship was used in determining the average interior chain length of boll weevil glycogens.

Exterior and Interior Linkage Analysis

Identification of isomers of the methylated glucosides released from methylated glycogen on acid hydrolysis and silanization (after careful consideration of their mutarotation properties) yielded data indicative of the interior linkages from which they were obtained. The α- and β-isomers were identified by their retention times on the non-polar liquid phase (SE-30) and by injection of authentic standards with the hydrolytic products from methylated glycogen.

The extent of mutarotation was determined by subjecting the methylated standards to optimal conditions (i.e., allowed to stand overnight at room temperature in water) and by hydrolyzing methylated disaccharides with the formic-sulfuric acid medium. Disaccharides were selected for this study based on their interior linkages and whether or not these linkages could be present in glycogen. The methylated, hydrolyzed, and silanized disaccharides and their linkages were maltose (α-1,4), cellobiose (β-1,4), and trehalose (α-1,1).

Disaccharides were methylated and hydrolyzed in the same manner as the glycogen samples except that the reaction time for methylation was reduced from five days to 18 hours. Acid hydrolysis was carried out the same as for the glycogens.

RESULTS

A total of ten experiments was conducted to determine the physical properties of trichloroacetic acid (TCA) and cold-water extracted (c.w.e.) boll weevil glycogens, so that they could be adequately compared to mammalian glycogens. The molecular weight, optical rotation, iodine binding power, and infra-red spectra were determined on glycogen extracted from eggs, larvae, and adult boll weevils. Other tests were performed to determine the purity (carbohydrate, protein, ash, and moisture) of each extracted material.

Chemical investigations were designed to elucidate the molecular structure and included experiments to define the average chain length, average exterior and interior chain lengths, and chain linkage of the component monosaccharide.

Since much glycogen is present in last instar larvae (Nettles and Betz, 1965), most of the glycogen was extracted from these insects. Early in the study one gram of glycogen was extracted with 5% TCA (and precipitation with ethanol) from 1,600 larvae. Repeated analyses of the TCAe. larval glycogen with the anthrone reagent of Carroll *et al.* (1956), yielded a maximum carbohydrate content of 67.5% with an average value of 66.3 $\pm$ 8%. Calculation of the glycogen content of the TCAe. material from the amount of glucose obtained on complete acid hydrolysis, gave a total carbohydrate content of 54.9% with glucose oxidase and 64.0% by the sulfuric acid test of Mendel *et al.* (1954).

During the preliminary analyses of the TCAe. glycogen, Bueding and Orrell (1965) showed that a mild extraction procedure caused less degradation to glycogen than extraction with either TCA or KOH. They showed that glycogen extracted by either hot alkali, TCA, or hot water has an average molecular weight 10 - 50 times lower than glycogen, of the same species, extracted by a cold-water procedure. Egg, larval, and adult boll weevil glycogens were cold-water extracted and compared to the TCAe. larval sample.

Since analyses of c.w.e. boll weevil glycogens with the anthrone reagent indicated a high degree of purity, additional analyses for non-carbohydrate constituents were not performed on the c.w.e. glycogens.

Physical analyses of the TCAe. larval glycogen were continued in an attempt to account for the remaining 33% of the sample. First experiments were designed to determine the amount of protein, if any, that had been extracted with the glycogen. The method of Moore and Stein (1954) was used on several concentrations of TCAe. boll weevil glycogen and averaged 5.7% protein. Glycine (1.399 μgN_2/ml) was used as the standard for this analysis and a standard curve, shown in Figure 4, was obtained at two concentrations. The Koch and McMeekin method (micro-Kjeldahl) averaged 6.3% protein for the TCAe. larval glycogen. Results of these two methods of protein determination are presented in Table 2.

Moisture content of the TCAe. and standard samples was determined by repeated weighings of approximately 50 mg samples held over phosphorus pentoxide in a desiccator. The results, shown in Table 3, were identical for glycogen obtained from both sources and averaged 10.4%.

Figure 4. Standard Curve of Glycine (1.399 μgN_2/ml) for Protein Determination of TCAe. Boll Weevil Glycogen.

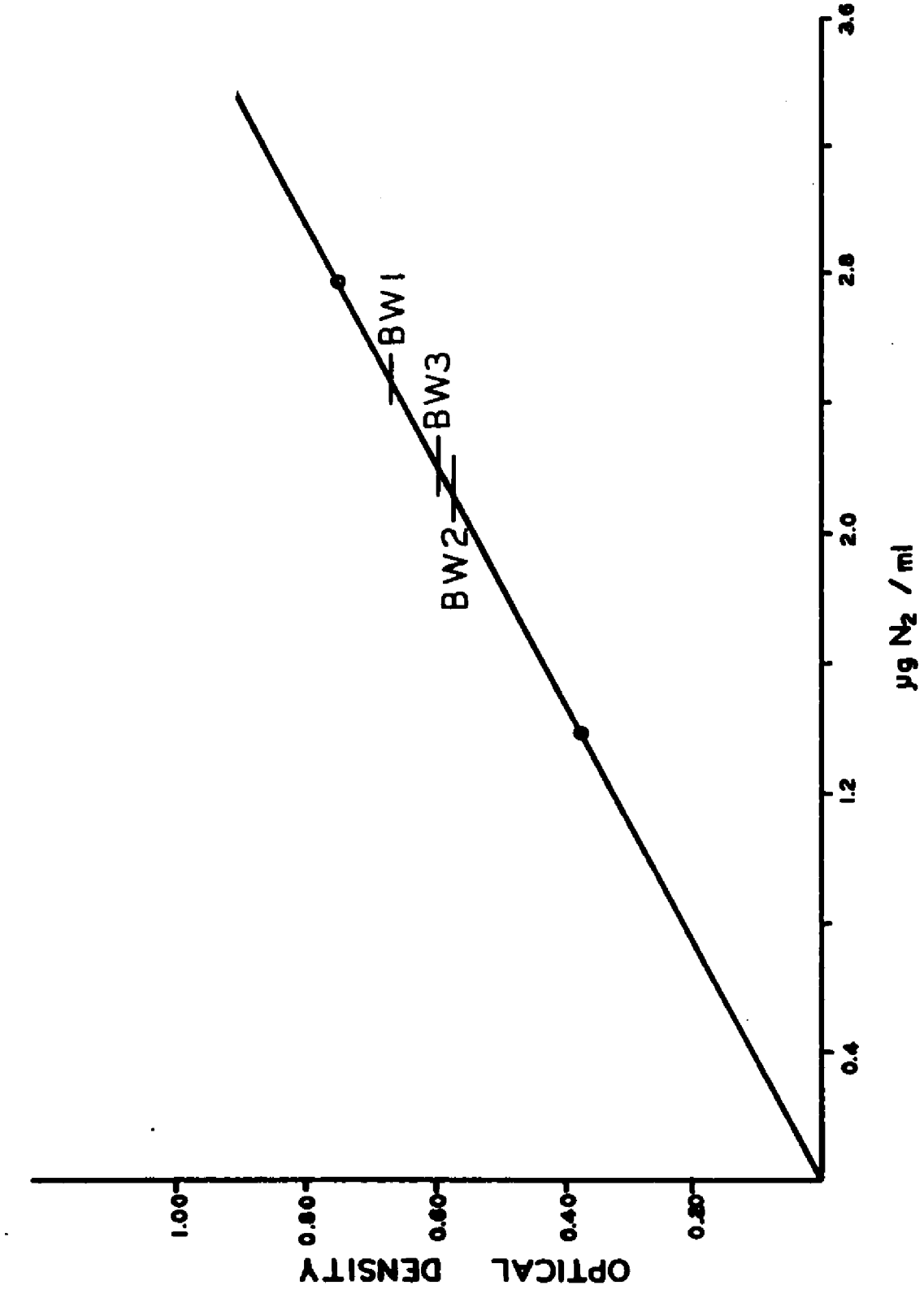

OPTICAL DENSITY
1.00
0.80
0.60
0.40
0.20
0.4
1.2
2.0
2.8
3.6
µg N2 / ml
BW1
BW3
BW2

Table 2. Protein Content of Boll Weevil Glycogen.

Sample	Glycogen[1/] (µg/ml)	Moore and Stein Method µgN_2/sample	% protein sample
Glycine (standard)	7.5	1.39	---
Boll Weevil, larvae TCAe. (#130)	500.0	10.28	6.41
Boll Weevil, larvae, TCAe. (#130)	250.0	8.88	5.55
Boll Weevil, larvae, TCAe. (#130)	50.0	8.00	5.00
		Koch and McMeekin Method	
Ammonium Sulfate (standard)		104.7	---
Boll Weevil, larvae, TCAe. (#130)	1000	10.12	6.33

[1/]Average of three determinations/sample.

Table 3. Moisture Content of Boll Weevil Glycogen

Sample	Glycogen (mg)	% moisture	Av.
Rabbit Liver (standard)	54.8	11.1	
	69.5	10.6	10.4%
	65.6	9.6	
	57.5	10.1	
Boll Weevil, larvae, TCAe. (#130)	79.2	10.7	
	86.8	9.7	10.4%
	54.3	10.7	
	58.0	10.5	

Table 4. Ash Content of Boll Weevil Glycogen.

Sample	Glycogen (mg)	% ash[1/]
Rabbit Liver (standard)	50.8	0.0
Boll Weevil, larvae, TCAe. (#130)	48.1	7.7

[1/]Dry weight basis, held at 600°C for 12 hours.

Ash content was calculated for the glycogens after ignition in a Muffle furnace at 600°C for 12 hours. Results of ash determinations on the TCAe. and standard glycogens, presented in Table 4 above, averaged 7.7% for the boll weevil sample. The standard glycogen was ash-free.

A TCAe. larval glycogen sample was chromatographed through a column of Sephadex G-200 gel to further determine its purity. Using the anthrone reagent, reducing material was determined before and after passage through the gel. Recovery was considered quantitative since 98.5% of the glycogen was recovered after passage through the Sephadex gel. Therefore, any contaminant in the glycogen had to be of a molecular weight exceeding 200,000, which is the limit of retention by Sephadex G-200.

High and low voltage paper electrophoresis of the carbohydrate portion was carried out and no detectible contaminants were observed for either voltage application.

The composition of TCAe. larval glycogen, c.w.e. larval glycogen, and standard (rabbit liver) glycogen are presented in Table 5.

The glycogen content of TCAe. larval glycogen, c.w.e. egg and larval glycogen, and standard glycogen by the anthrone method is presented in Table 6. The lowest value obtained for any c.w.e. glycogen was considerably higher than either the TCAe. or standard glycogens. The purity of these glycogens was also obtained by calculation of the glucose yielded on complete acid hydrolysis with 1N sulfuric acid at 100°C for 2.5 hours. After hydrolysis, the pH of the samples was adjusted to 6.0 - 7.0 with barium hydroxide and centrifuged. After adding water, the barium sulfate precipitates were reacted with the anthrone reagent to determine whether glucose was trapped by the precipitating barium sulfate. The average loss using this precipitation amounted to 27μg/mg sample.

An aliquot of each supernatant was pipetted into a centrifuge tube, and glycogen analyses were performed on the ethanol precipitate to determine the amount of unhydrolyzed glycogen per sample. The average amount of unhydrolyzed glycogen was 2μg/mg sample.

The supernatants were analyzed for glucose content with anthrone, glucose oxidase, and by gas-liquid chromatography (GLC) of the trimethylsilyl derivative. These results are presented in Table 7 and show that glucose oxidase gave lower values than either the anthrone method or the gas-liquid chromatographic method. As calculated from these glucose hydrolyzates, the glycogen content of TCAe., c.w.e. egg and c.w.e. larval samples was 62.2%, 97.7%, and 99.3%, respectively.

Table 5. Glycogen Content Obtained by Three Extraction Procedures.

	Cold Water[1/]	5% TCA[2/]	KOH[3/]
Carbohydrate Content (anthrone)	100%	67%	88%
Protein	--	5.8%	--
Moisture	--	10.4%	10.4%
Ash	--	7.7%	--

[1/]Larval Glycogen

[2/]Larval Glycogen

[3/]Commercial: Rabbit Liver Glycogen

Table 6. Carbohydrate Content of Boll Weevil and Standard Glycogens by the Anthrone Reaction.

Sample	Glycogen [1/]
Boll Weevil, eggs, c.w.e. (#112, MeOH)	97.7
Boll Weevil, eggs, c.w.e. (#133, EtOH)	98.4
Boll Weevil, larvae, c.w.e. (#120)	102.7
Boll Weevil, larvae, c.w.e. (#103)	98.3
Boll Weevil, larvae, TCAe. (130)	66.3
Rabbit Liver, standard, KOHe.	87.6

[1/]Average of 3 determinations/sample.

Table 7. Glucose Released on Complete Acid Hydrolysis as Determined by the Anthrone Reagent, Glucose Oxidase, and Gas-Liquid Chromatography. 1/

Sample	Anthrone	Glucose Oxidase	GLC 2/
Boll Weevil, larvae, TCAe. (#130)	691.5	548.5	596.8
Boll Weevil, eggs, c.w.e. (#112, MeOH)	1085.0	874.0	990.0
Boll Weevil, larvae, c.w.e. (#120)	1103.0	958.0	1211.1

1/ Micrograms of glucose/1 mg sample.

2/ Non-polar (SE-52) Liquid Phase only.

The optical rotation was determined for the TCAe. and standard glycogens but because of the excessive opalescence of the c.w.e. glycogens, specific rotation could not be determined. The optical rotation of the standard glycogen (literature value = + 178°) was +174.0° and for boll weevil glycogen was +200.0°. A sample of maltose (+131.7°) was used as an additional standard material for calibration of the polarimeter and the observed value was +131.3°. These results are presented in Table 8.

The absorption spectrum of boll weevil glycogen was measured as the iodine-complex with a Beckman DU and a recording Beckman DB spectrophotometer. The region of maximum absorption with the Beckman DU was 370 mμ for the boll weevil and standard glycogens as their iodine complexes in an iodine-potassium iodide medium. The spectra

Table 8. Optical Rotation of Boll Weevil Glycogen.

Sample	Conc. (g./100 ml)	α_D^{20} 1/	$[\alpha]_D^{20}$
Maltose (standard)	4.0	+5.25°	+131.3° 2/
Rabbit Liver, standard KOHe.	1.0	+1.74°	+174.0° 3/
Boll Weevil, larvae, TCAe. (#130)	0.6	+0.81°	+200.0°

1/Average of 3 determinations.

2/Literature value = +131.7°.

3/Literature value = +178.0°.

are presented in Figure 5, where amylopectin was used as an iodine binding polysaccharide (for comparative purposes).

The iodine-complexes obtained with the recording DB are presented in Figure 6. The regions of maximum absorption for TCAe. larval glycogen, c.w.e. egg, larval, and adult glycogen, and standard glycogen were 383 mμ, 400 mμ, 395 mμ, and 375 mμ , respectively.

Infra-red absorption spectra for TCAe. and c.w.e. glycogens were determined by analyzing potassium bromide pellets with a Beckman IR-5 spectrophotometer. The regions of absorption, shown in Table 9, are characteristic for glycogen and were identical to the absorption areas of the standard. Characteristic infra-red absorption bands corresponded to primary, secondary, and tertiary alcohol structures, as well as to that characteristic of α-D-glucopyranose structures. These spectra, shown in Figure 7, show a major area of

Figure 5. Absorption Spectra of the Iodine Complexes of Boll Weevil and Standard Glycogens Obtained with a Beckman DU Spectrophotometer.

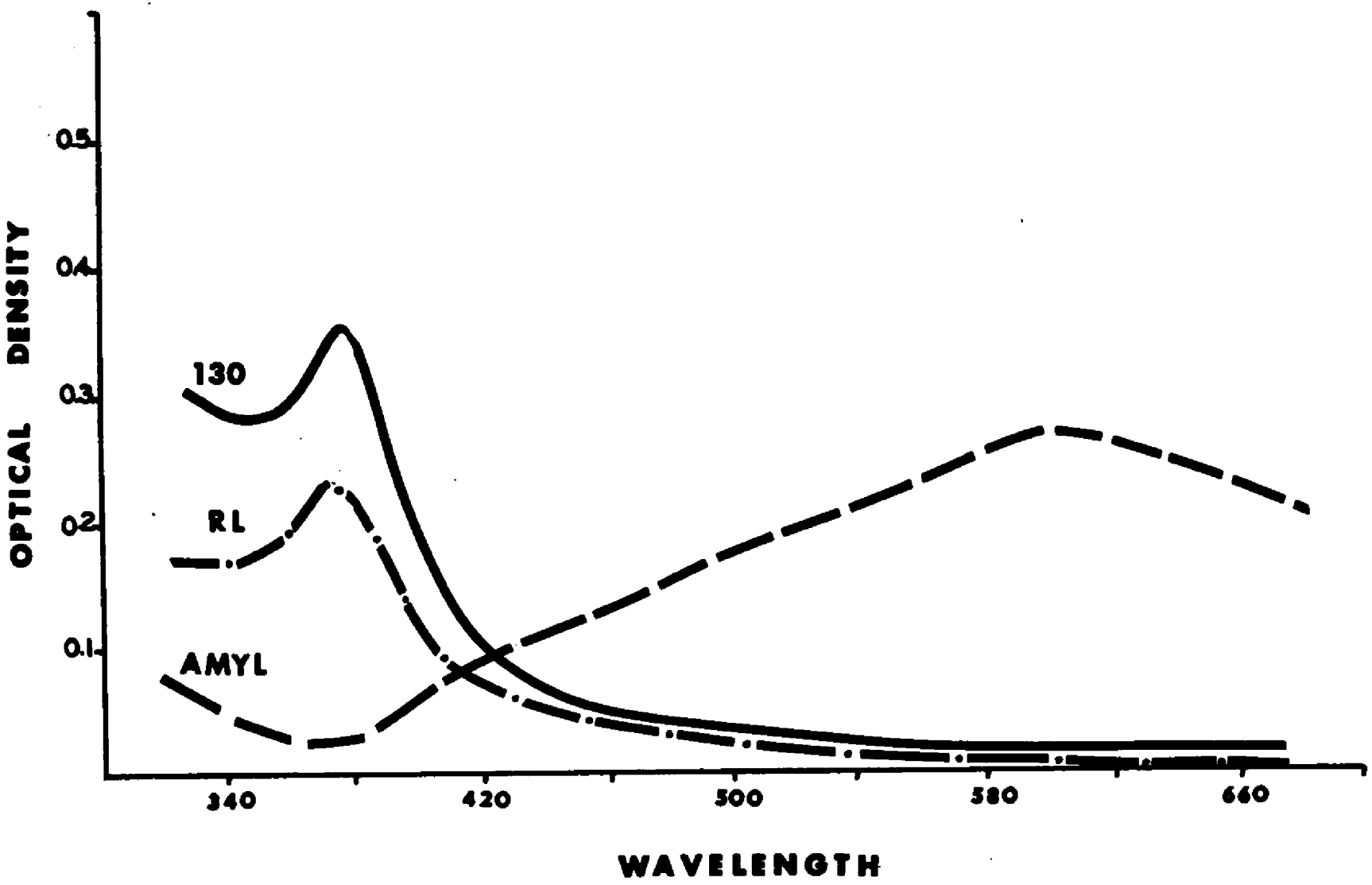

OPTICAL DENSITY
0.5
0.4
0.3
0.2
0.1
130
RL
AMYL
340
420
500
580
660
WAVELENGTH

Figure 6. Absorption Spectra of the Iodine Complexes of Boll Weevil and Standard Glycogens Obtained with a Recording Beckman DB Spectrophotometer.

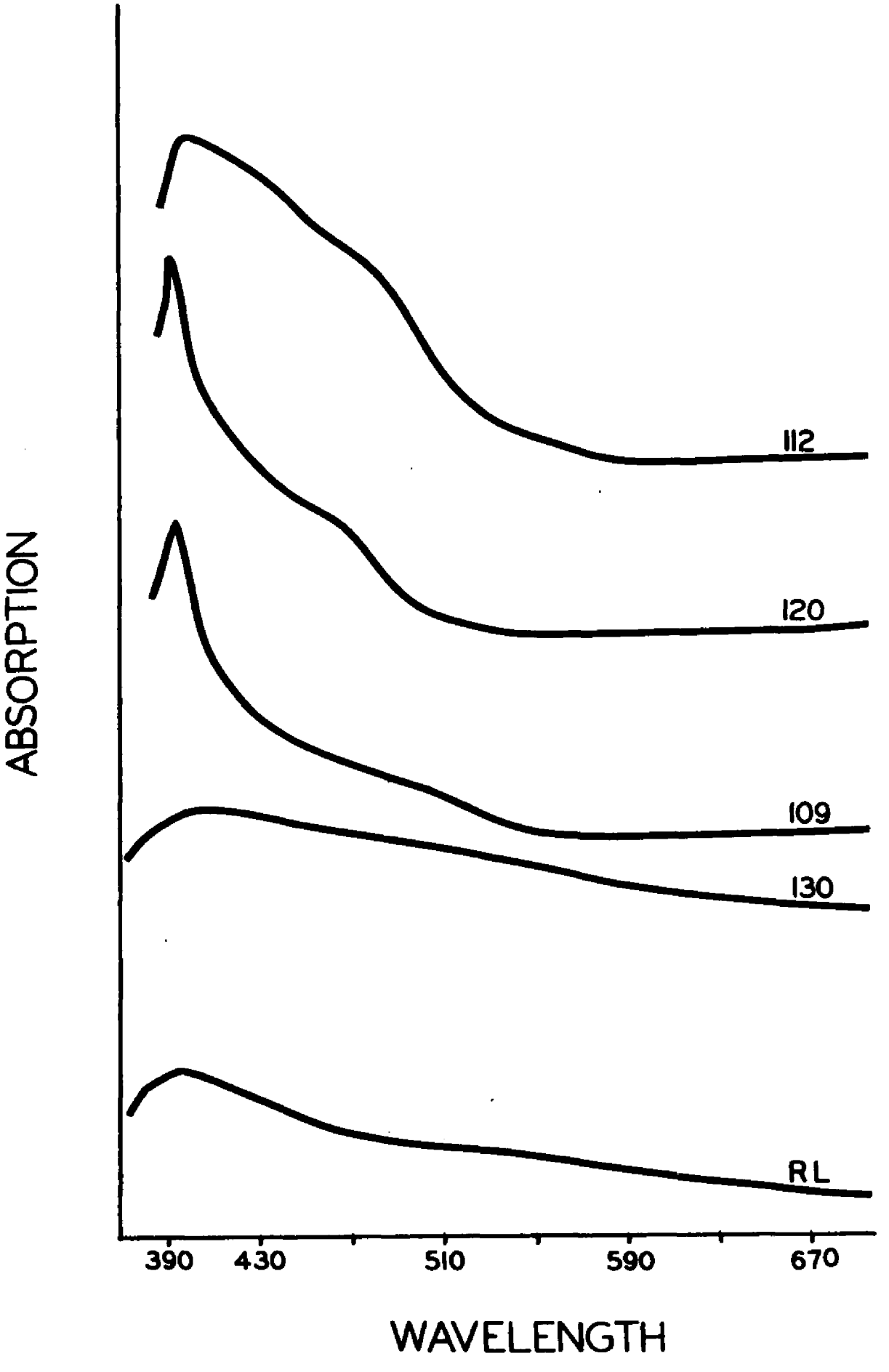
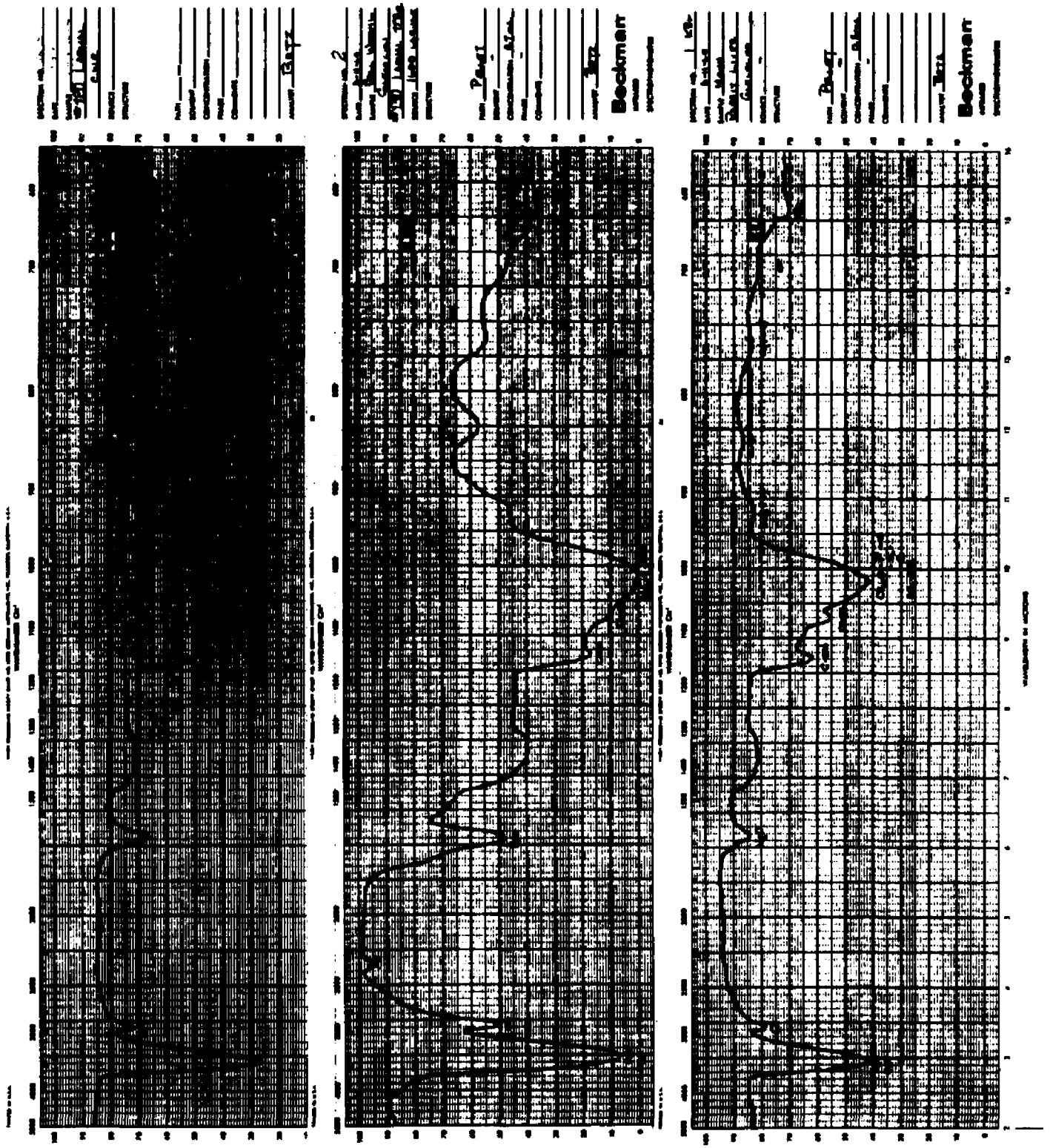

ABSORPTION
112
120
109
130
RL
390
430
510
590
670
WAVELENGTH

Table 9. Regions of Absorption of Boll Weevil Glycogens in the Infrared.

Sample	Major		Minor	
	microns	cm^{-1}	microns	cm^{-1}
Rabbit Liver, standard	2.9	3400	3.4	2580
	9.8	1020	6.2	1625
			7.4	1350
			8.7	1150
			9.3	1080
			10.8	930
			11.9	835
			13.2	760
			15.1	665
Boll Weevil, larvae, TCAe. (#130)	2.9	3400	3.4	2580
	9.8	1020	6.2	1625
			7.5	1350
			8.7	1150
			9.3	1080
			10.8	930
			12.0	835
			13.2	760
			15.1	665
Boll Weevil, larvae, c.w.e. (#120)	2.9	3400	3.4	2580
	9.8	1020	6.1	1640
			7.5	1350
			8.7	1150
			9.3	1080
			10.8	930
			11.8	850
			13.2	760
			15.1	665

Figure 7. Infra-red Spectra of Boll Weevil and Standard Glycogens Obtained with a Beckman IR-5.

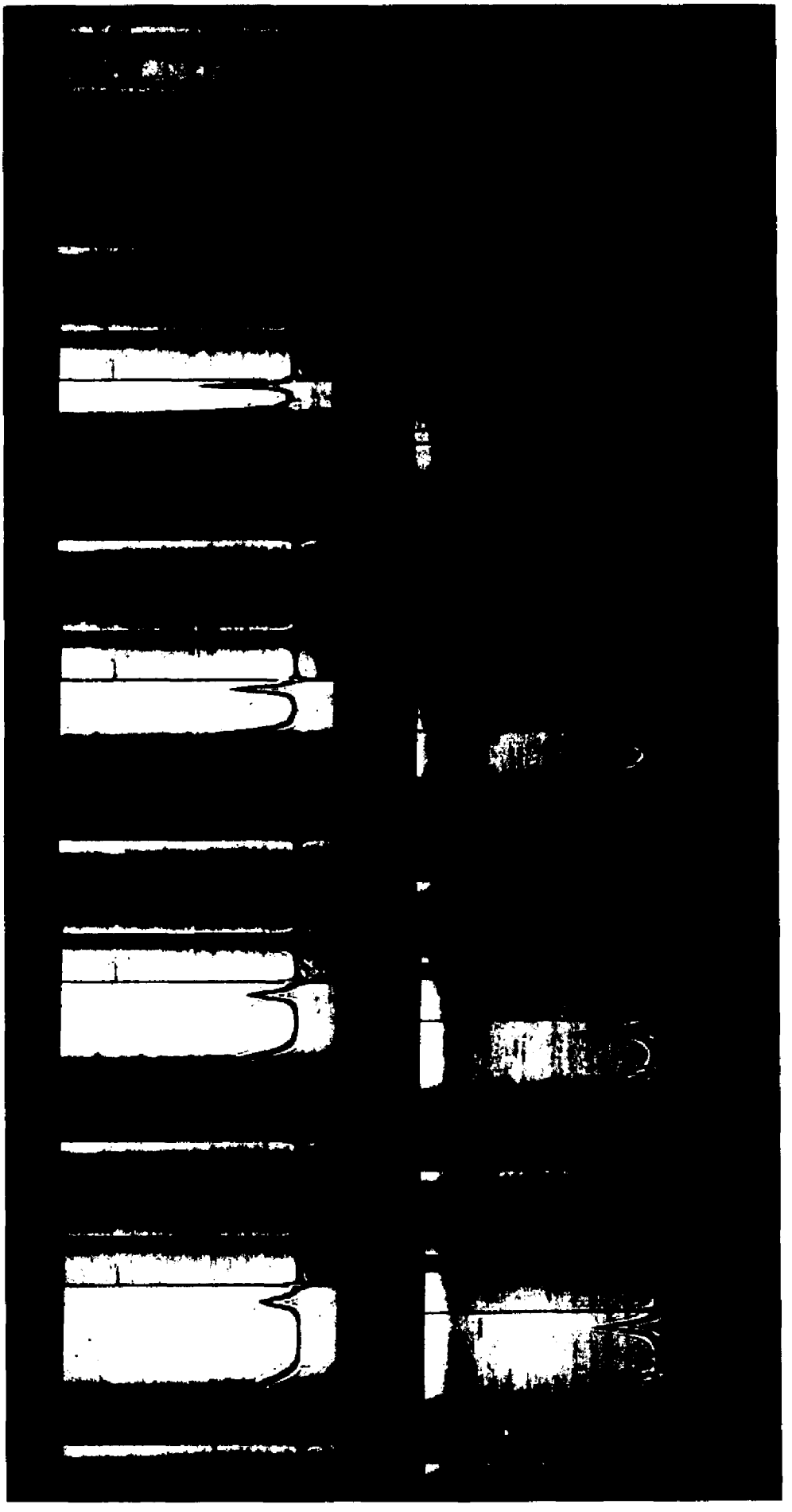


absorption (2.9 microns) characteristic of hydroxyl compounds. The absorption peak at 6.2 microns was identified as water, most of which appears during the preparation of the KBr pellets. The infra-red spectra of boll weevil glycogen were identical to that of rabbit liver glycogen based on the Sadtler code obtained from the literature.

Molecular weight determinations were carried out at the Johns Hopkins School of Medicine in Baltimore, Maryland by Mr. Stanley A. Orrell. Samples, analyzed by their characteristic sedimentation patterns in the analytical ultra-centrifuge, included both the TCAe. larval glycogen and c.w.e. samples. The molecular weight data of adult boll weevil glycogen was attempted, but the sample size was not sufficient for complete sedimentation. All samples were subjected to the same run speed (9340 rpm), temperature (20°C), phase plate angle (45°), and acceleration (1 min. 10 sec. linear rate). Concentrations were approximately 10 mg/ml in water; the final results were corrected to zero concentration by an IBM computer.

Molecular weight sedimentation data were obtained for c.w.e. larval glycogen (reared on the normal diet B and on a high sugar diet), c.w.e. adult glycogen, and TCAe. larval glycogen. Several samples of egg glycogen were subjected to ultracentrifugation, but the plates obtained from the Schlieren optical system were rejected by the IBM system (i.e., negative values were obtained). Corrected curves for egg glycogens could not be calculated because of the exceedingly high molecular weight material in the second fraction. The contact prints for egg glycogens in Figure 8 show two distinct (and completely

Figure 8. Contact Prints Obtained by Ultracentrifugation of Egg Glycogen Samples.



separated) regions of molecular weight. The photographs are reversed in that the pictures were made from the right, the first picture being made three minutes after beginning the linear acceleration. The second picture was made four minutes after the first; the third, four minutes after the second, etc. The darkening toward the left of each frame (high molecular weight region), very prominent in the egg glycogen samples, was caused by the high turbidity of the egg glycogens. The prints also show the complete separation of the low and high molecular weight materials which, according to Orrell (personal communication), was strikingly different than all glycogens analyzed by this procedure. In fact, there is no similarity between boll weevil egg glycogen and all other defined glycogens.

The remarkably heavy molecular weight material in the egg glycogen interfered with computation of the sedimentation curves since the expression programmed into the computer had a maximum limit of approximately 1000S. The high molecular weight fraction of the egg glycogen was estimated to be three to five times as great as other glycogens. The low molecular weight region appeared to be nearly the same for egg and other defined glycogens. The elaborate corrections handled by the computer were concentration dependent and the distribution curves obtained were corrected to zero concentration. The high viscosity (resulting from the higher molecular weight material) of the egg samples interfered with the distribution of the material at zero concentration. Series concentrations with appropriate analytical expressions were tried unsuccessfully. Boll weevil egg glycogen was found to have the

highest weight average molecular weight (from the contact prints only) of all glycogens analyzed to date.

Curves of sedimentation data were obtained for c.w.e. larval glycogen; the larvae were reared on regular diet B and on diet B with 20% sucrose added. These sedimentation data are presented in Figures 9 and 10; c.w.e. larval glycogen on regular diet B is denoted 103 and c.w.e. larval glycogen reared on the high sugar diet is denoted 101 (a replicate of 120). All curves are comparable, since they have been corrected to finite concentration and differences between these curves are a result of their structure and not their concentration. The high sugar diet did not substantially affect either molecular weight region of the c.w.e. larval glycogen.

The sedimentation curves showed that the high sucrose diet produced a slightly higher weight average molecular weight for the lower region and an extended curve in the higher region. This, however, was compensated for in the normal diet which showed quantitatively more of the lower and higher molecular weight materials of weight average less than the high sucrose diet.

The c.w.e. adult glycogen gave the sedimentation pattern presented in Figure 11. The low and high molecular weight regions exhibited higher weight averages but were quantitatively less than the other c.w.e. glycogens.

A strikingly different sedimentation pattern was obtained for the TCAe. larval glycogen. Figure 12 shows the pattern for TCAe. glycogen, completely devoid of any high molecular weight material.

Figure 9. Sedimentation Data Collected for c.w.e. Boll Weevil Larval Glycogen.

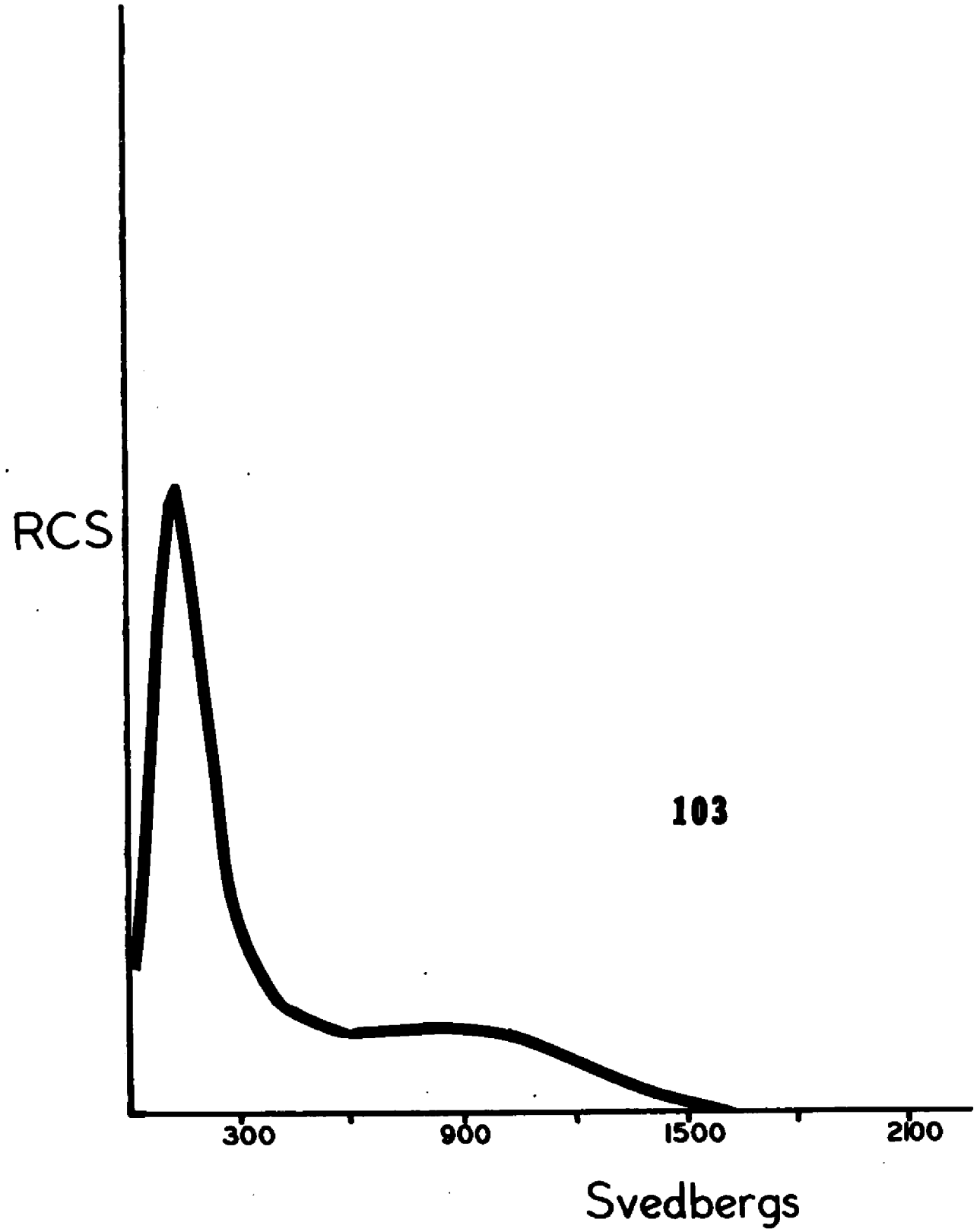

RCS
103
300
900
1500
2100
Svedbergs

Figure 10. Sedimentation Data Collected for c.w.e. Boll Weevil Larval Glycogen (High Sucrose Diet).

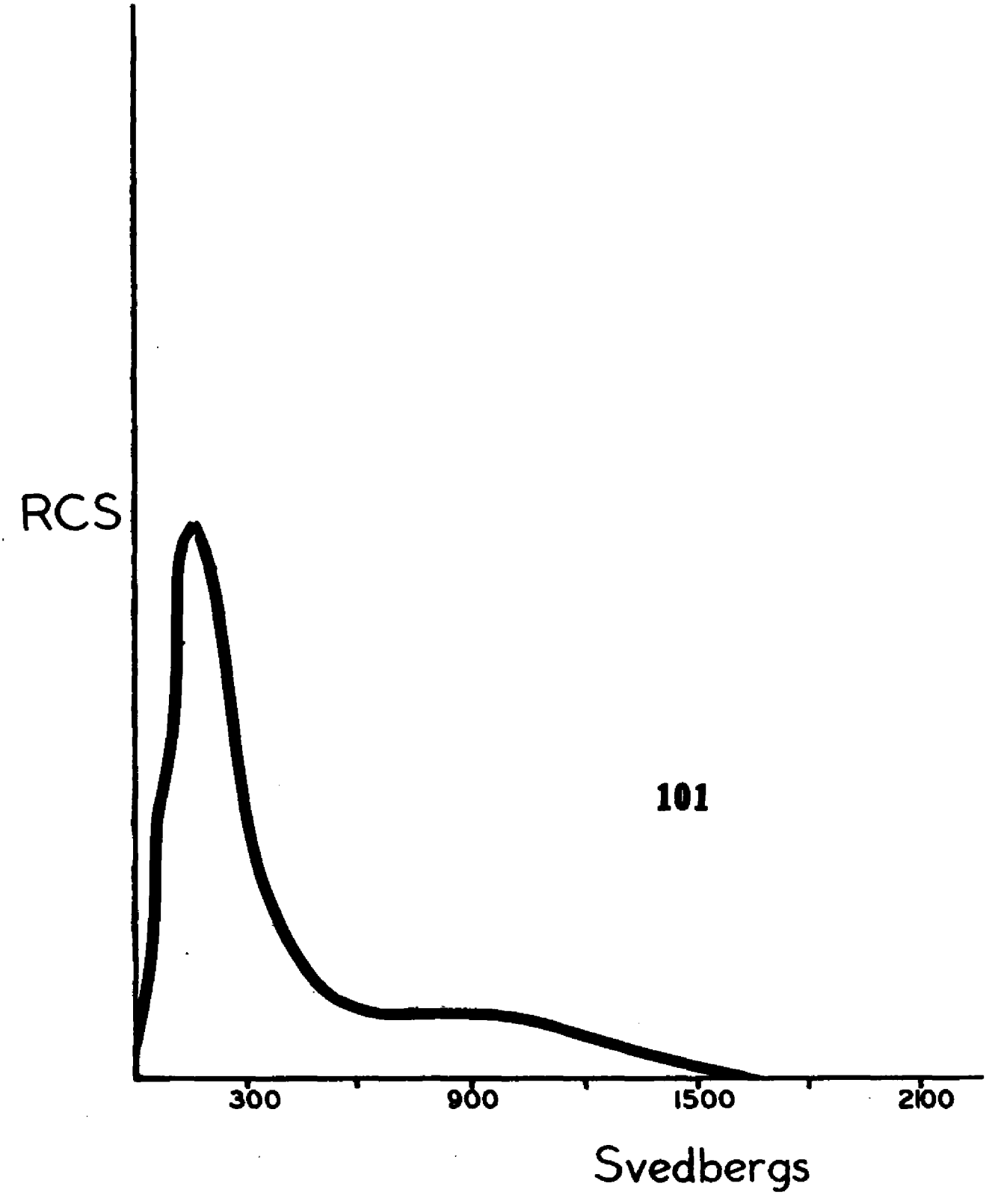

RCS
101
300
900
1500
2100
Svedbergs

Figure 11. Sedimentation Data Collected for c.w.e. Boll Weevil Adult Glycogen.

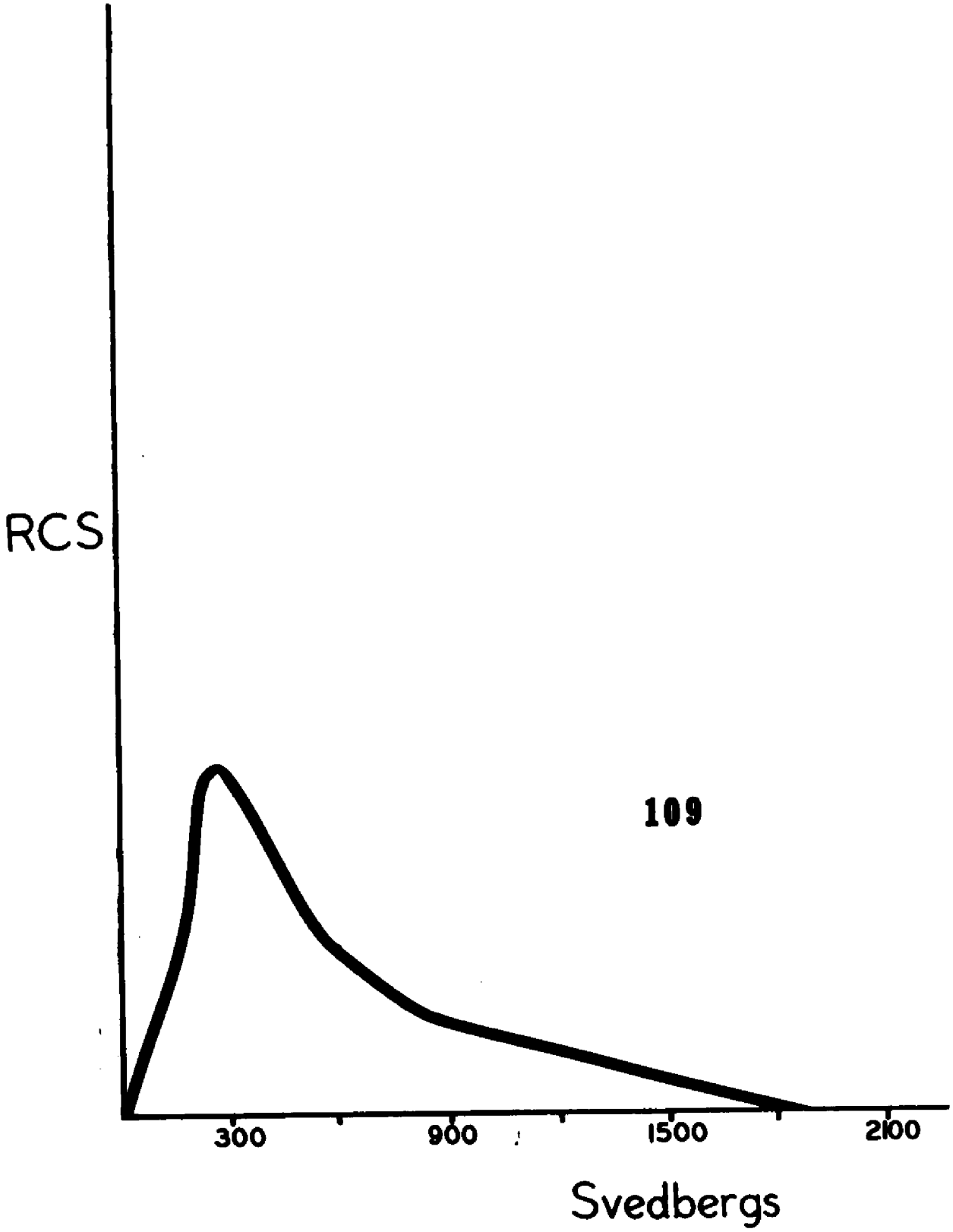
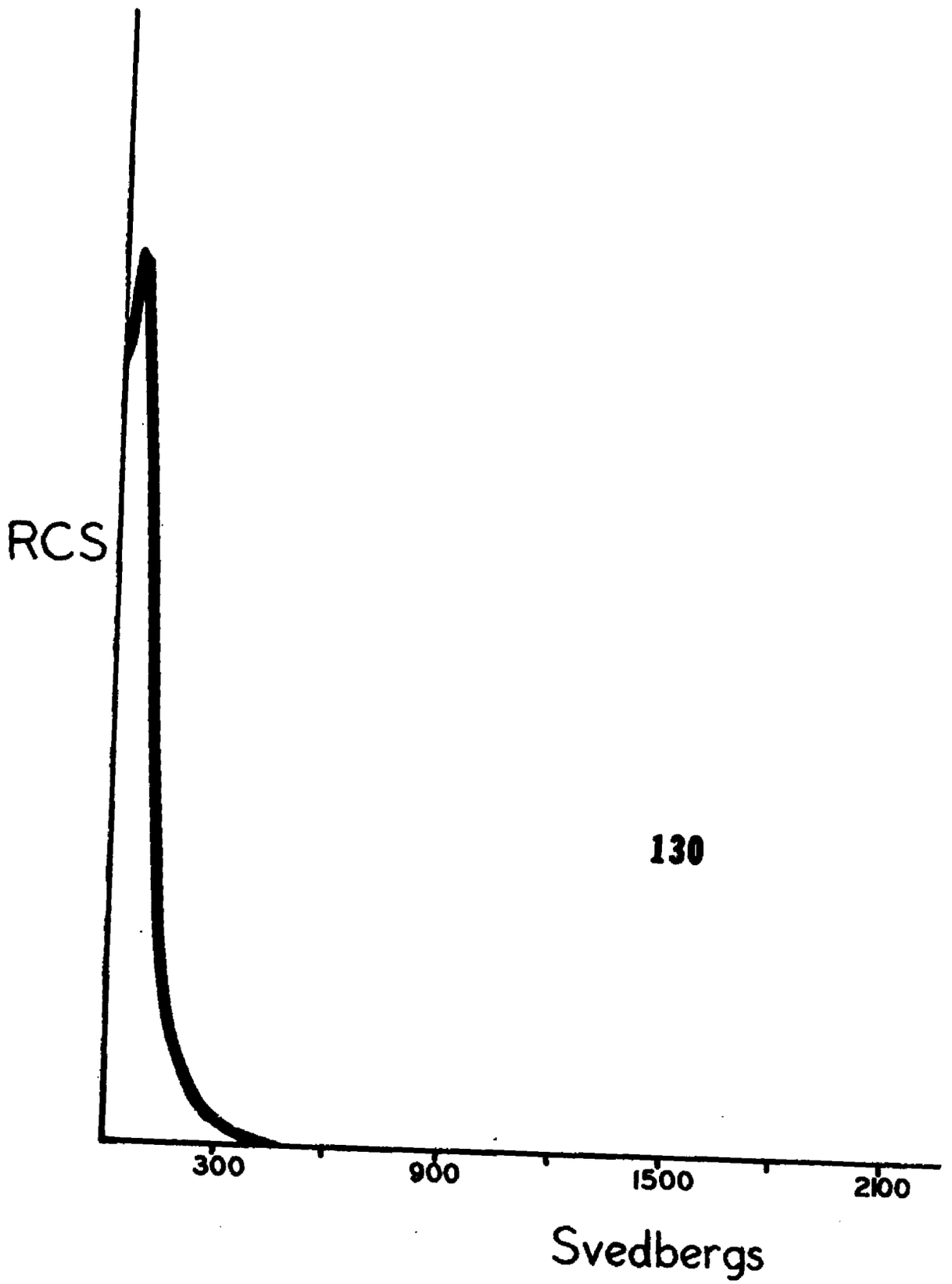

RCS
109
300
900
1500
2100
Svedbergs

Figure 12. Sedimentation Data Collected for TCAe. Boll Weevil Larval Glycogen.


RCS
130
300
900
1500
2100
Svedbergs

It was apparent from these data that the high molecular weight was completely degraded during the extraction procedure. This observation paralleled that of Orrell and Bueding (1965) for rabbit liver glycogen using these two extraction procedures. According to Bueding (personal communication), the decrease in high molecular weight material from c.w.e. glycogen was not completely accounted for by an increase in the low molecular weight TCAe. component. It is suspected that some high molecular weight material was degraded to an ethanol-soluble saccharide fragment. Figure 12 also showed that the low molecular weight material had a weight average less than any c.w.e. material.

A summary of the sedimentation data for boll weevil glycogens is presented in Table 10. All c.w.e. glycogens have patterns that suggested two regions of molecular weight. The lower molecular weight regions (100-300 svedbergs) differed only quantitatively among the c.w.e. samples. The higher molecular weight regions, exhibited by all c.w.e. samples, appeared in the region of 900-1500 svedbergs; with the exception of the egg samples, the highest weight average pattern was obtained from the c.w.e. adult glycogen. Both the TCAe. boll weevil glycogen and the KOHe. rabbit liver glycogen gave sedimentation data characteristic of lower molecular weight only.

The physical properties of TCAe. and c.w.e. boll weevil glycogens, presented in Table 11, are compared with those of the KOHe. standard. The molecular weight data show that the c.w.e. glycogens always had an average molecular weight greater than the TCAe. or

Table 10. Molecular Weight Sedimentation Data for Boll Weevil Glycogens. [1/]

Sample	Low Mol. Wt. Region	High Mol. Wt. Region
Boll Weevil, Larvae c.w.e. (#120)	200	900-1200
Boll Weevil, Larvae, c.w.e. (#103)	100-200	900-1000
Boll Weevil, Adults, c.w.e. (#109)	300	1200-1500
Boll Weevil, Larvae, TCAe. (#130)	100	-
Rabbit Liver, Standard, KOHe.[2/]	150-175	-

[1/]Data expressed in Svedbergs (1 Svedberg = 1 x 10^{-13}cm/dyne sec).
[2/]Orrell and Bueding (1965).

Table 11. Comparison of Physical Properties of Glycogens Obtained by Three Extraction Procedures.

	c.w.e.	TCAe.	KOHe.
Molecular Wt. (Svedbergs)	N/A[1/] (eggs) 150-200; 900-1200 (larvae 300;1200-1500 (adults)	50-100 (larvae)	150-175[2/]
Optical Rotation	N/A[3/]	+200°	+178°
Maximum absorption in the infra-red	1020, 3400 cm^{-1}	1020, 3400 cm^{-1}	1020, 3400 cm^{-1}
Maximum absorption of the I_2-complex	400 mμ (eggs) 395 mμ (larvae) 395 mμ (adults)	383 mμ	375 mμ

[1/]Molecular weight of egg glycogen too high for IBM computers to calculate.

[2/]Orrell and Bueding, 1965.

[3/]Highly opalescent solutions were too dense for determing $[\alpha]_D^{20}$

KOHe. samples. On the other hand, the regions of maximum absorption for the three extraction procedures in the infra-red and of the iodine-complexes in the visible range were essentially the same.

Boll weevil glycogens were subjected to vigorous acid hydrolysis, neutralized, and the hydrolyzate analyzed for monosaccharide content. Total losses during neutralization with barium hydroxide including the amount of unhydrolyzed glycogen amounted to 29 µg/mg sample.

Initially, these hydrolyzates were evaporated to dryness under reduced pressure and were taken up with a known volume of water. An aliquot of each sample was removed and subjected to thin-layer chromatography (TLC) in three solvent systems. Of the solvent systems tested for adequate movement (R_f) and separation of hexoses, n-butanol:acetone:water (4:4:1) gave good separations and was the most rapid. The slowest, n-butanol:acetic acid:water (4:1:1), gave the best resolution of hexoses. A third solvent system, selected as an additional test, was ethyl acetate:isopropanol:water (3:3:1).

As determined by TLC in three solvent systems, only glucose was released after complete acid hydrolysis. These results are presented in Table 12. The resolution and movement of glucose in these systems was improved by preparing the silica gel H solid support, as a slurry in 0.1N boric acid solution. Plates from all solvent systems were developed with a spray reagent of diphenylamine:aniline:phosphoric acid (5:5:1;2% w/v in acetone:2% w/v in acetone:85% ortho-phosphoric acid). Glucose gave a characteristic blue color with this spray system.

Table 12. Glucose Released on Complete Acid Hydrolysis as Determined by Thin-Layer Chromatography.[1/]

	Solvent System		
	1[2/]	2[3/]	3[4/]
Sample	R_f	R_f	R_f
Glucose	0.39	0.31	0.26
Boll Weevil, larvae TCAe. (#130)	0.39	0.31	0.27
Boll Weevil, eggs, c.w.e. (#112, MeOH)	0.38	0.29	0.26
Boll Weevil, larvae, c.w.e. (#120)	0.38	0.35	0.25

[1/] Silica Gel H, prepared as 0.1N boric acid slurry.

[2/] Butanol:acetone:water (4:4:1).

[3/] Ethyl acetate:isopropanol:water (3:3:1).

[4/] Butanol:acetic acid:water (4:1:1).

After qualitative analysis of glucose from the hydrolyzates by thin-layer chromatography, quantitative analysis was carried out with glucose oxidase, according to the method in the data sheet furnished by the manufacturer (Worthington Biochemical Co., Freehold, New Jersey). The limit of sensitivity was 50μg glucose and the enzymic preparation (Glucostat Special) was free of contaminating carbohydrases. The results of these analyses are presented in Table 13. All values were lower than those obtained by anthrone or gas-liquid chromatography (Table 7).

Table 13. Glucose Released on Complete Acid Hydrolysis as Determined by Glucose Oxidase.

Sample	Glucose Content 1/ %
Boll Weevil, larvae, TCAe. (#130)	54.8
Boll Weevil eggs, c.w.e. (#112, MeOH)	87.4
Boll Weevil, larvae c.w.e. (#120)	95.8

1/Average of three samples, two determinations/sample.

The hydrolyzates were analyzed qualitatively and quantitatively simultaneously by gas-liquid chromatography of the trimethylsilyl derivatives. Several of the hydrolyzates were taken to dryness under reduced pressure at 30°C, the residue dissolved with 1 ml anhydrous pyridine and reacted with the silanization agents. After standing at room temperature for 10 min., an aliquot of this mixture was analyzed by gas-liquid chromatography using polar (20% diethylene glycol succinate, EGS) and non-polar (3% SE-52) liquid phases. Qualitative analysis of the peaks obtained was based on the retention times (t_R) for the trimethylsilyl derivatives of dextrose (α-glucose), β-glucose, and the mutarotated forms of these sugars. The sugars were allowed to mutarotate by placing 20 mg samples of the sugars in water and standing overnight at room temperature under toluene.

The results of GLC of these sugars are presented in Table 14 for the non-polar liquid phase, SE-52. Table 15 shows the results of

Table 14. Glucose Released on Complete Acid Hydrolysis as Determined by Gas-Liquid Chromatography on a Non-Polar Liquid Phase.[1]

Sample	α-Glucose		β-Glucose	
	t_R[2]	%[3]	t_R	%
α-Glucose	16.0	99+	--	--
β-Glucose	16.1	2.7	23.5	97.3
Dextrose (M)[4]	16.1	40.2	23.9	59.8
Boll Weevil, larvae, TCAe. (#130)	16.2	42.6	24.1	57.4
Boll Weevil, eggs, c.w.e. (#112, MeOH)	16.1	42.5	24.2	57.5
Boll Weevil, larvae, c.w.e. (#120)	16.4	37.4	24.3	62.6

[1] Liquid phase = 3% SE-52 on acid and base washed, silanized, Chromport XXX. (Inlet 220°, Col. 188°, Det. 215°C).

[2] t_R = Retention time.

[3] Average of four samples, three determinations/sample.

[4] Dextrose (M) = α-Glucose allowed to mutarotate overnight in water.

gas-liquid chromatography of these sugars for the polar liquid phase, EGS. The results of GLC of the hydrolyzates from boll weevil glycogen are also presented in Tables 14 and 15. Shown in these tables, the observed equilibrium for the mutarotated sugar derivatives was approximately 40% α and 60% β; the observed literature values for the α- and β-isomers are 36% and 64%, respectively.[1]

[1] J. S. Fruton and S. Simmonds, General Biochemistry (Second Edition; New York: John Wiley and Sons, Inc., 1959), p. 406.

Table 15. Glucose Released on Complete Acid Hydrolysis as Determined by Gas-Liquid Chromatography on a Polar Liquid Phase. [1/]

Sample	α-Glucose		β-Glucose	
	t_R [2/]	% [3/]	t_R	% [3/]
α-Glucose	4.2	99+	--	--
β-Glucose	4.2	2.4	7.6	97.6
Dextrose (M) [4/]	4.3	40.2	7.8	59.8
Boll Weevil, larvae, TCAe. (#130)	4.1	40.6	7.6	59.4

1/ Liquid phase = 20% EGS on acid and based washed, silanized, Chromport XXX. (Inlet 225°, Col. 145°, Det. 210°C).

2/ t_R = Retention time.

3/ Average of four samples, three determinations/sample.

4/ Dextrose (M) = α-Glucose allowed to mutarotate overnight in water.

Typical gas chromatograms of the standard and standard mutarotated sugars are presented in Figure 13 for the non-polar liquid phase. A comparison of the chromatograms obtained for mutarotated β-glucose and a boll weevil hydrolyzate is presented in Figure 14, which also shows the characteristic retention times for the polar and non-polar liquid phases.

The amount of glucose (α+β) present in each hydrolyzate by gas-liquid chromatography was determined quantitatively by plotting a standard curve of the response (integration units) given by known amounts of α-and β-glucose. These results are presented in Table 7; the standard curves obtained for α-and β-glucose are presented in Figure 15.

Figure 13. Gas Chromatograms of Standard β-Glucose, Dextrose (α-Glucose), β-Glucose (Mutarotated) and Dextrose (Mutarotated) on a Non-Polar Liquid Phase (3% SE-52).

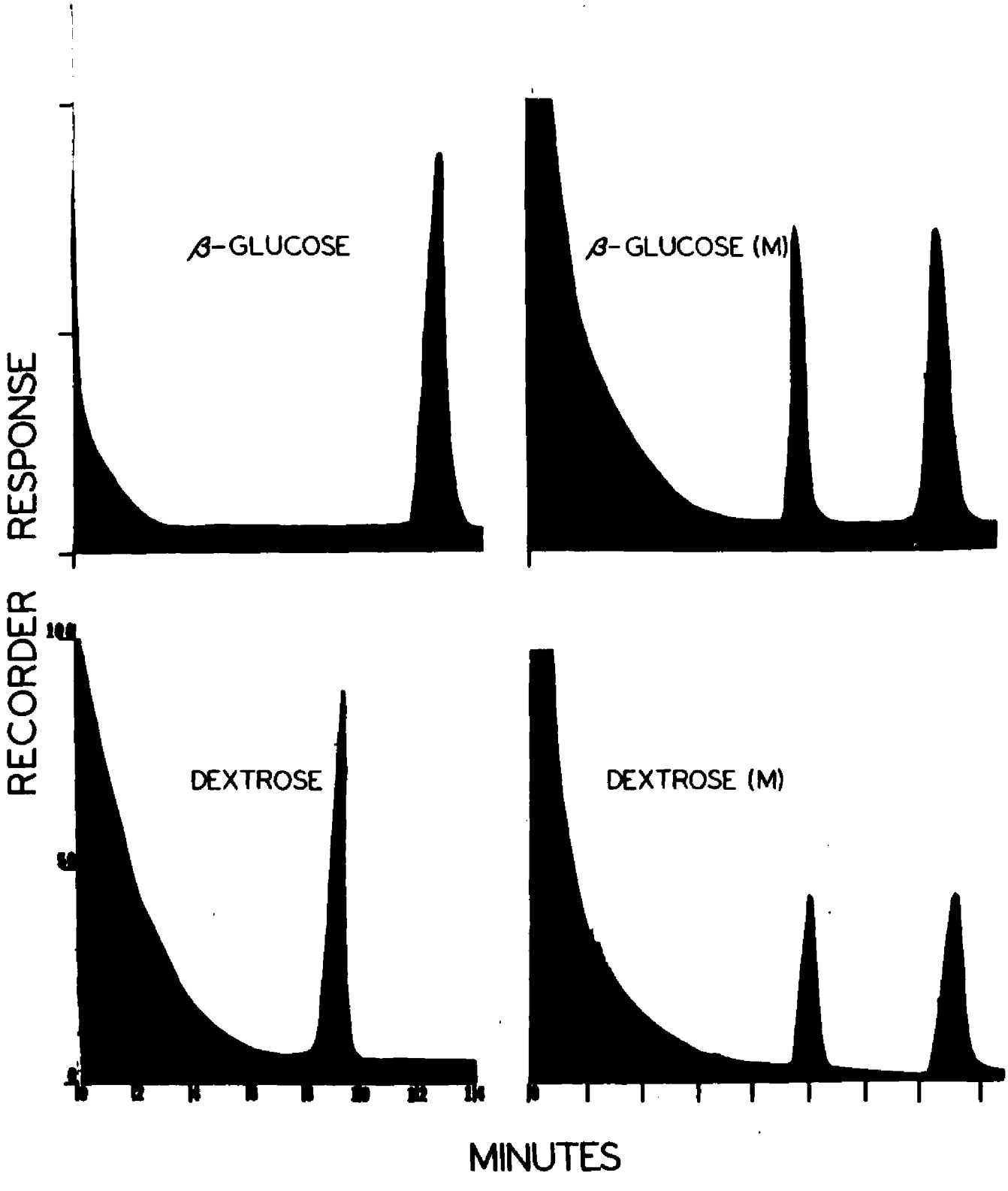

β-GLUCOSE
β-GLUCOSE (M)
DEXTROSE
DEXTROSE (M)
RECORDER RESPONSE
MINUTES

Figure 14. Gas Chromatograms of β-Glucose (Mutarotated) and Boll Weevil Acid Hydrolyzate (Mutarotated) on a Polar and a Non-Polar Liquid Phase.

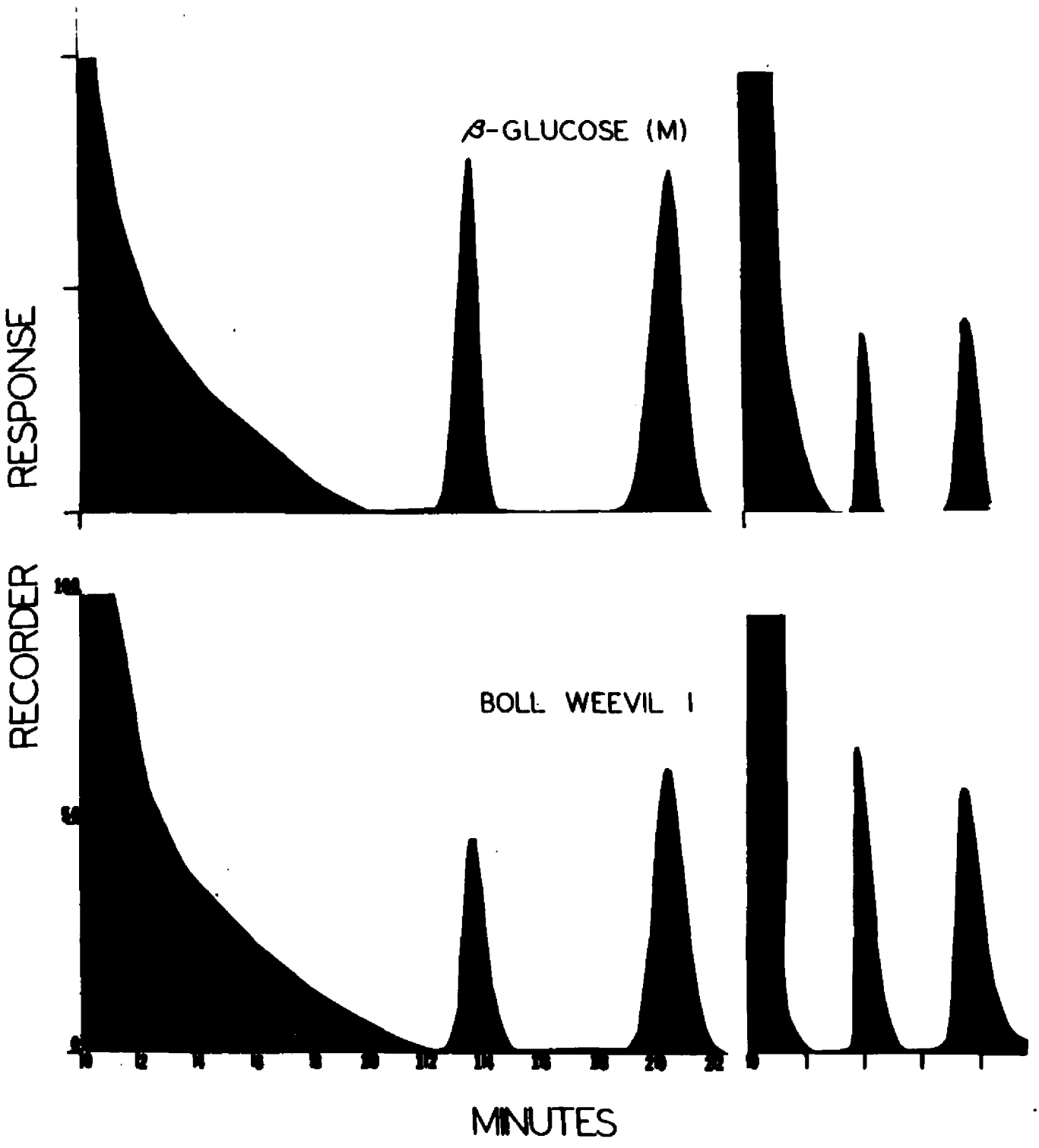
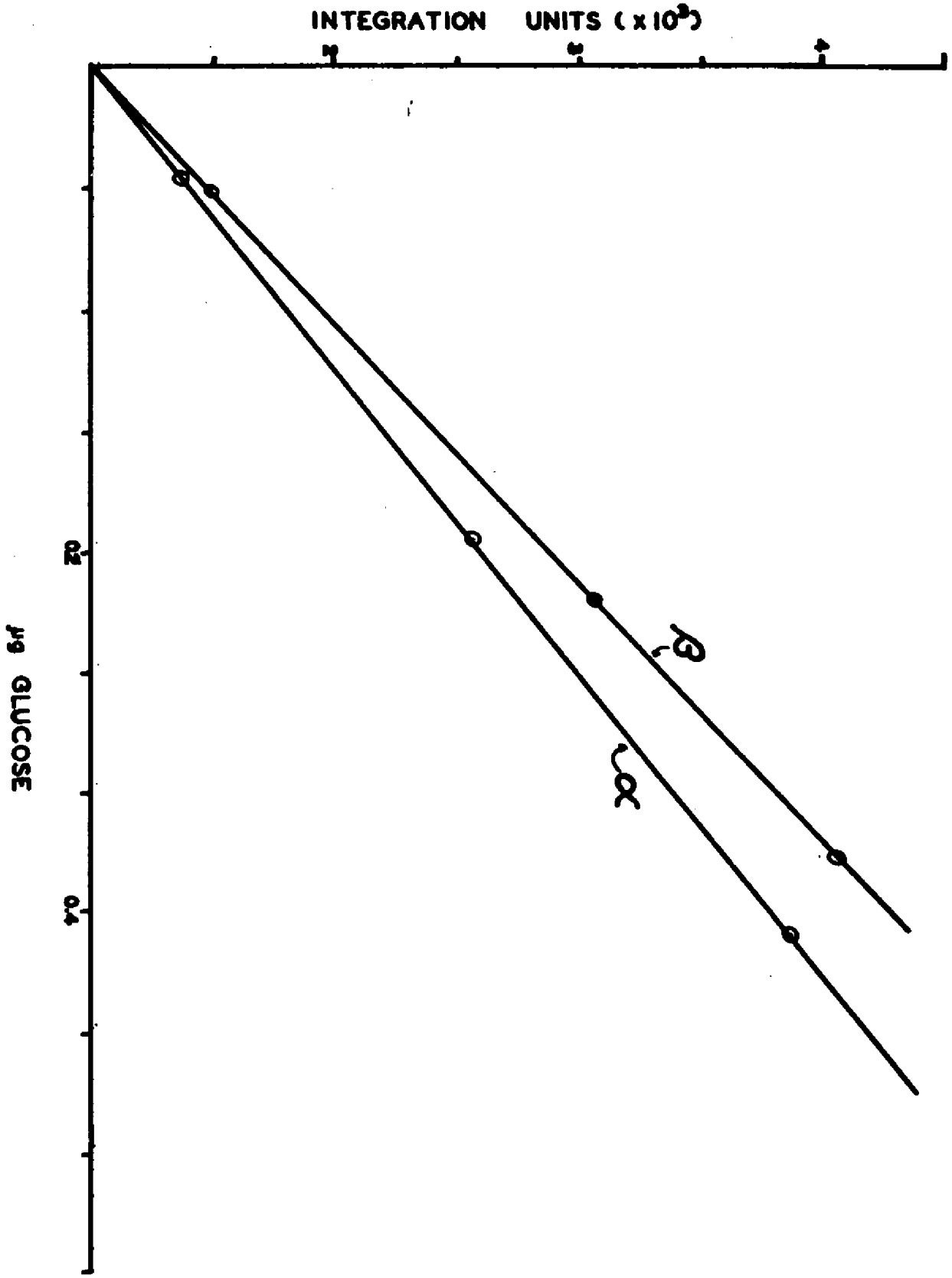

3% SE-52
20% EGS
β-GLUCOSE (M)
RESPONSE
RECORDER
BOLL WEEVIL I
MINUTES

Figure 15. Standard Curve Obtained for Injected Amounts of Standard α - and β -Glucose.


INTEGRATION UNITS (x10³)
mg GLUCOSE
20
40
β
α

Using sterile techniques, the per cent cleavage by β-amylase was determined for boll weevil glycogens. Samples were incubated with three times recrystallized β-amylase, which cleaves α-1,4 linked glucans from the non-reducing end group and removes maltose, one molecule at a time, until a branch point is reached. Near the branch point, α-1,6, the action of β-amylase is terminated. The remaining structure is called the limit dextrin (L.D.) and the per cent cleavage by β-amylase results in a β-amylolysis L.D. Beta-amylase was obtained commercially (in saturated ammonium sulfate) with an activity of 600u/mg and a turnover number of 250,000 linkages/molecule.

After stabilizing the temperature of the enzyme-buffer mixture (20°C), glycogen solutions were injected (through Millipore filters) into each sealed tube. After incubation for four hours at 20°C, the tubes were evaporated to dryness under reduced pressure at 30°C, and taken up with a small volume of water. An aliquot of this solution was chromatographed on thin layers of silica gel H; these results are presented in Table 16. When developed with the spray reagent, maltose and the β-amylolysis limit dextrin were detected. Rough quantitation of the amount of maltose present (and therefore the per cent β-amylolysis) was calculated from a standard curve of maltose obtained from a recording densitometer. A recorder trace and the standard curve are presented in Figure 16.

The remainder of each sample was placed on a column of Sephadex G-200 gel and eluted with distilled water. Since the molecular size of the L.D. did not allow penetration of the gel, the L.D. was eluted within the first 15 fractions (50-2 ml fractions were

Table 16. Results of Thin-Layer Chromatography of Maltose Obtained from β-Amylase Digestion of Glycogens. [1/]

Compound	Solvent System 1[2/] R_f	Solvent System 2[3/] R_f
Maltose (STD)	0.34	0.19
Boll Weevil, eggs, c.w.e. (#112, MeOH)	0.33	0.17
Boll Weevil, larvae, c.w.e. (#120)	0.33	0.17
Boll Weevil, larvae, TCAe. (#130)	0.33	0.17
RL, standard, KOHe.	0.33	0.17

[1/]Silica Gel H, prepared as 0.1N Boric acid slurry.

[2/]Butanol:Acetone:Water (4:4:1).

[3/]Butanol:Acetic acid:Water (4:1:1).

collected). The maltose remained on the column; its elution time was determined by the void volume (V_o) of the gel. Best separations and elution times were obtained with a V_o of 100 ml.

One ml aliquots of each tube were removed and reacted with the anthrone reagent to determine the amount of maltose and L.D. separated by the gel. The per cent maltose yielded by the boll weevil glycogens is presented in Table 17; their elution patterns are presented in Figure 17. The remaining aliquots of separated maltose and L.D. were lyophilized and chromatographed by thin-layer techniques. The maltose

Figure 16. Densitometer Trace for Known Amounts of Maltose and the Standard Curve Obtained from the Trace.

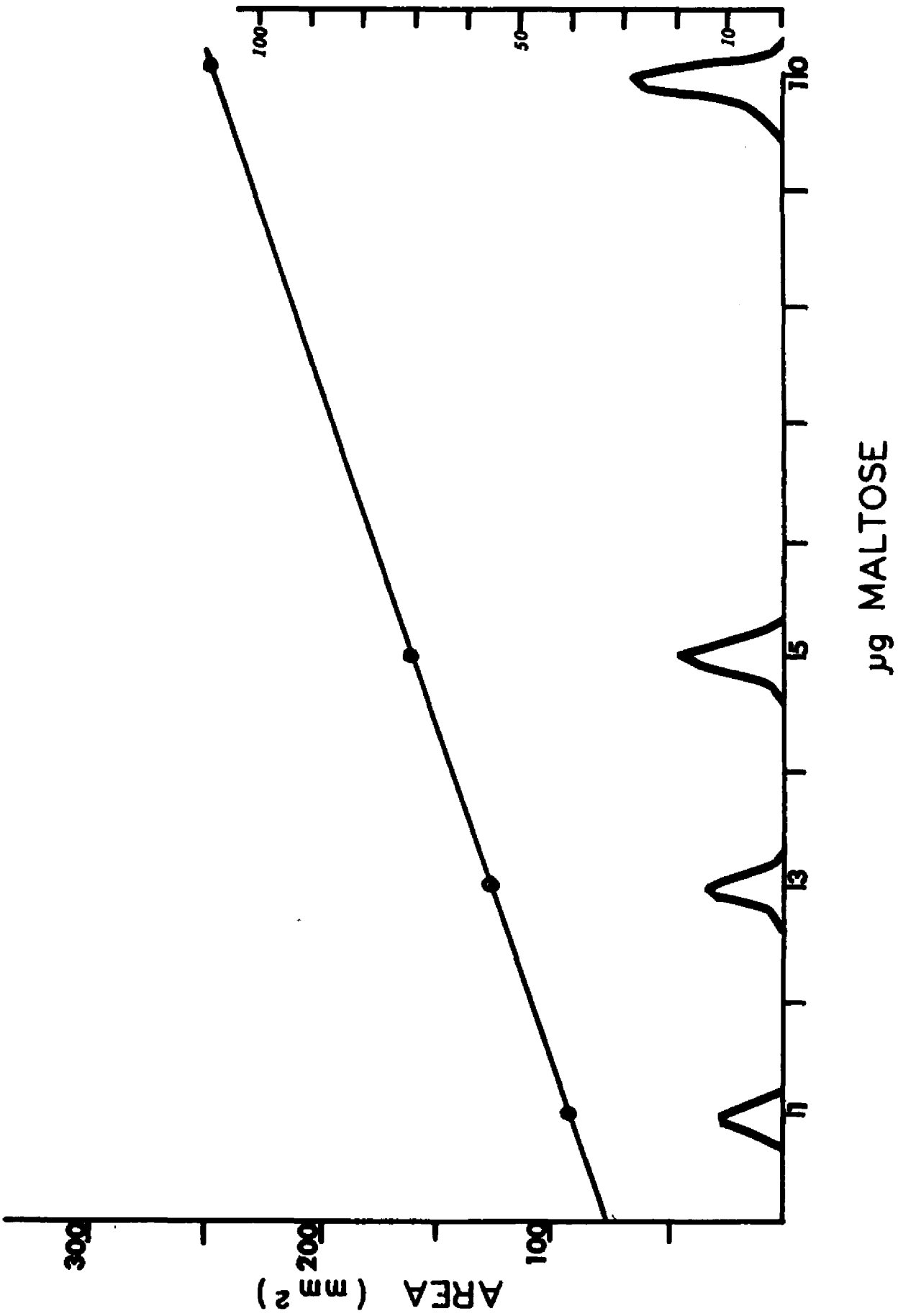

AREA (mm²)
300
200
100
100
50
10
1
3
5
10
µg MALTOSE

Table 17. Action of β-Amylase and Phosphorylase b on Boll Weevil and Standard Glycogens.

Sample	% β-Amylolysis[1/]	% Phosphorolysis[2/]
Boll Weevil, egg, c.w.e. (#112, MeOH)	62.4	30.4
Boll Weevil, eggs, c.w.e. (#133, EtOH)	61.7	--
Boll Weevil, larvae, c.w.e. (#120)	51.2	--
Boll Weevil, larvae, TCAe. (#130)	59.0	39.6
Boll Weevil, adults, c.w.e. (#109)	--	26.5
Rabbit Liver, standard, KOHe.	52.4	--

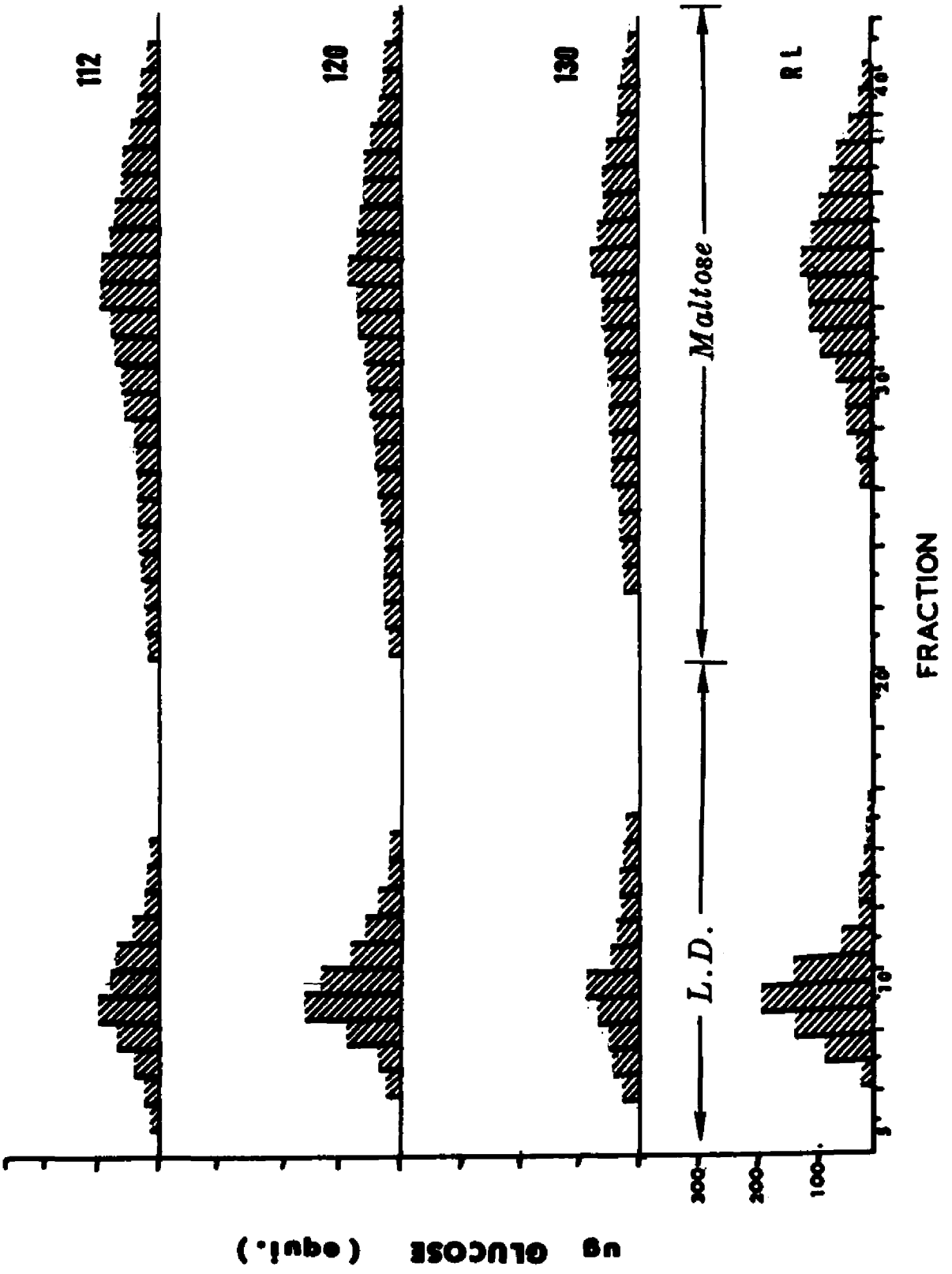
[1/]Anthrone positive material as maltose.

[2/]Enzymic determination of Glucose-1-Phosphate produced by the action of phosphorylase alone.

fraction was free of contaminating carbohydrates by thin-layer chromatography and similar treatment of the L.D. fractions resulted in detection of a single spot at the origin. These materials were stored in the freezer at -20°C until linkage analyses could be performed.

Since the per cent maltose was determined from the per cent of anthrone positive material in the second series of fractions eluted from the Sephadex gel, analyses were made to obtain the amount of glucose yielded by a known amount of maltose. One hundred μg of maltose yielded an average of 108 μg of glucose when reacted with

Figure 17. β-Amylolysis Limit Dextrins of Boll Weevil and Standard Glycogens.


112
120
130
R L
L. D.
Maltose
FRACTION
µg GLUCOSE (equi.)
300
200
100
10
20
30
40

the anthrone reagent. The factor for converting glucose to maltose was 0.93. The results of β-amylolysis are presented as the amount of maltose obtained from a given amount of glycogen.

Boll weevil glycogens were also subjected to exhaustive degradation by phosphorylase b. Phosphorylase, in the presence of inorganic phosphate, catalyzes the conversion of α-1,4 linked glucose units to glucose-1-phosphate. For enzymic determination, the glucose-1-phosphate produced is converted to glucose-6-phosphate in the presence of phosphoglucomutase, nicotinamide-adenine dinucleotide phosphate (NADP), and catalytic amounts of glucose-1,6-diphosphate. Spectrophotometric determination of the glucose-1-phosphate was based on the molar extinction coefficient of reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$), which is 6.22×10^6 cm^2/mole for $NADPH_2$ at 340 mμ (Horecker and Kornberg, 1948). The results of phosphorylase limit dextrin determinations are presented in Table 17.

The average chain length (CL) for boll weevil glycogens was determined by enzymic (Bueding and Hawkins, 1964) and non-enzymic (Betz, Nettles, and Novak, unpublished data) methods. The CL was calculated from data obtained during the phosphorylase limit dextrin assay and these results are presented in Table 18. The per cent branch points are indicative of the degree of branching and were obtained from the ratio of free glucose to glucose-1-phosphate produced by the total degradation of glycogen. The CL was calculated from the degree of branching; the CL for 8.0% branch points was 12.5 glucose units.

Table 18. Enzymic Determination of the Average Chain Lengths for Boll Weevil and Standard Glycogens.

Sample	% Branch points	Average chain length	Average 1/ exterior chain length	Average 2/ interior chain length
Boll Weevil, Adults, c.w.e. (#109)	9.1	11.0	6.9	4.1
Boll Weevil, eggs, c.w.e. (#112, MeOH)	8.0	12.5	7.7	4.8
Boll Weevil, larvae, TCAe. (#130)	8.1	12.4	8.9	3.5
Rabbit Liver, standard, KOHe.3/	-	11.0	7.5	3.5

1/Calculated by % CL + 4

2/Calculated by CL - ECL = ICL

3/Kjølberg and Manners, 1962

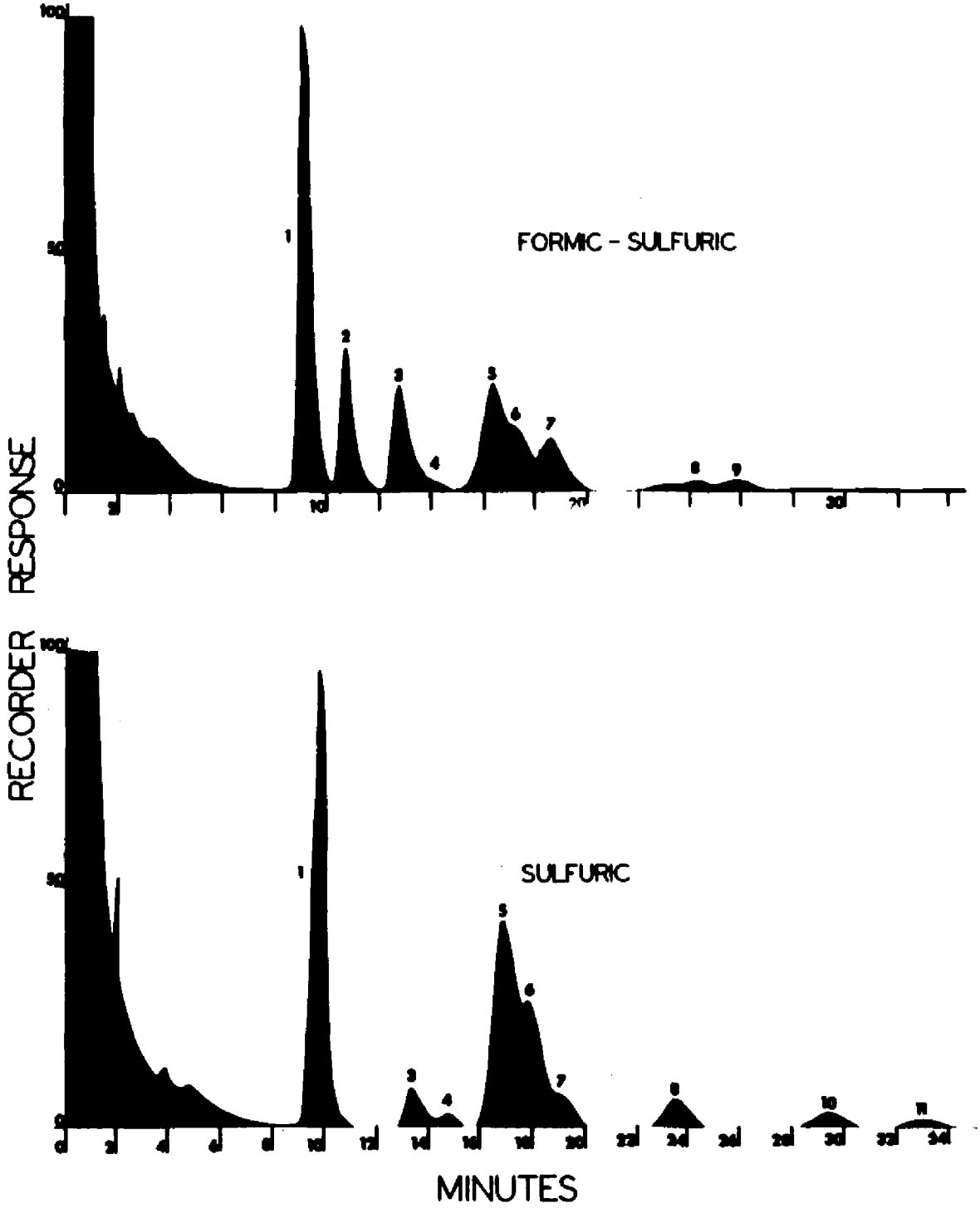
The CL was also determined by a new technique which involved the methylation, hydrolysis, silanization, and gas-liquid chromatography of the methylated-silanized products. Gas chromatography of methylated hexoses resulted in poor separation and the sugars could not be easily quantitated. Resolution was greatly improved by the addition of the alkyl radical, trimethylsilyl (TMS), to the free hydroxyl group (s) of each sugar. The trimethylsilyl derivatives of the methylated hexoses were prepared quantitatively and the gas-liquid chromatography substantially improved.

Earlier attempts to methylate glycogen appeared to be unsuccessful. It was suspected, at that time, that methylation was not proceeding to completion, and the resulting methylated glucose was only

methyl glucopyranoside. However, it was found that methylation was proceeding to completion and during hydrolysis, most of the methoxy radicals were being removed. At that time, 0.5N sulfuric acid was being used to hydrolyze the methylated glycogen. A modification of the method of Bouveng and Lindberg (1965) proved to be the most successful means of preventing demethylation.

To obtain complete methylation, the reaction time was increased to five days at 30°C. Acid hydrolysis with formic acid (90%) was carried out at 75°C for two hours, followed by addition of an equal volume of 0.1N sulfuric acid and the mixture was held overnight at 75°C. To determine the relative amounts of demethylation and degradation during acid hydrolysis samples of methylated sugars were subjected to three types of acid hydrolysis procedures. The method of Bouveng and Lindberg (1965) was compared to the method of Croon et al. (1960) and to the 0.5N sulfuric acid method. After methylation, a sample of maltose was split; half of the sample was hydrolyzed with the formic-sulfuric method and the remainder by the sulfuric acid method of Croon et al. (1960). The resulting hydrolytic products were separated by gas-liquid chromatography after neutralization with the combined Dowex resins. Unfortunately, the hydrolytic products from formolysis remained in the formic-sulfuric acid medium several days prior to deionization (designated as uncontrolled formolysis). The gas-chromatograms obtained (Figure 18) showed extensive demethylation for both hydrolytic methods; the sulfuric acid (Croon) method exhibited a larger amount of degradation. However, formolysis produced an additional peak (#2, Figure 18) of retention time = 11.0

Figure 18. A Comparison of Gas Chromatograms Obtained from Uncontrolled Formolysis and Sulfuric Acids During the Hydrolysis of Octamethyl Maltose.


FORMIC - SULFURIC
SULFURIC
RECORDER RESPONSE
MINUTES

min. that was not present in the hydrolytic products from the Croon method. This additional peak has been obtained by others working with gas-liquid identification of methylated unsilanized dextrins following hydrolysis by formic acid (Kircher, 1960; Adams and Bishop, 1960). The retention times reported for this compound by Kircher (1960) were between that obtained for methyl-2,3,4,6-tetra-O-methyl-D-glucoside and the α- and β-isomers of methyl-2,3,4-tri-O-methyl-D-glucoside. Kircher (1960) identified the sugar as 2,3,4-tri-O-methyl levoglucosan (1,6-anhydro-2,3,4-tri-O-methyl-β-D-glucopyranose). This artifact (peak #2, Figure 18) produced during formolysis behaved identically (based on retention data) to the levoglucosan reported by Kircher (1960). However, Kircher (1960) used fully methylated hydroxyethylcellulose on Chromosorb as liquid-phase and solid support respectively, so it was not identified as such during this study (SE-30; Chromport XXX).

By controlling the method of Bouveng and Lindberg (1965), gas chromatograms were obtained which still exhibited the artifact but the extensive demethylation and degradation was absent (Figure 19). A modification of the formolysis method (i.e., lower temperature and shorter exposure to the acidic mixture) eliminated the artifact and demethylation or degradation of a methylated rabbit liver glycogen standard (Figure 20). A sample of octamethyl maltose was also subjected to hydrolysis by 0.5N sulfuric acid at 100°C (conditions used for acid hydrolysis of glycogen). Figure 21 shows the extensive fragmentation, as shown by the peaks prior to tetramethylglucopyranoside (t_R = 8.5 min.). An indication of the amount of

Figure 19. A Comparison of Gas Chromatograms (TMS Derivatives) Obtained by Controlled Formolysis, Uncontrolled Formolysis, and Sulfuric Acid During the Hydrolysis of Octamethyl Maltose.

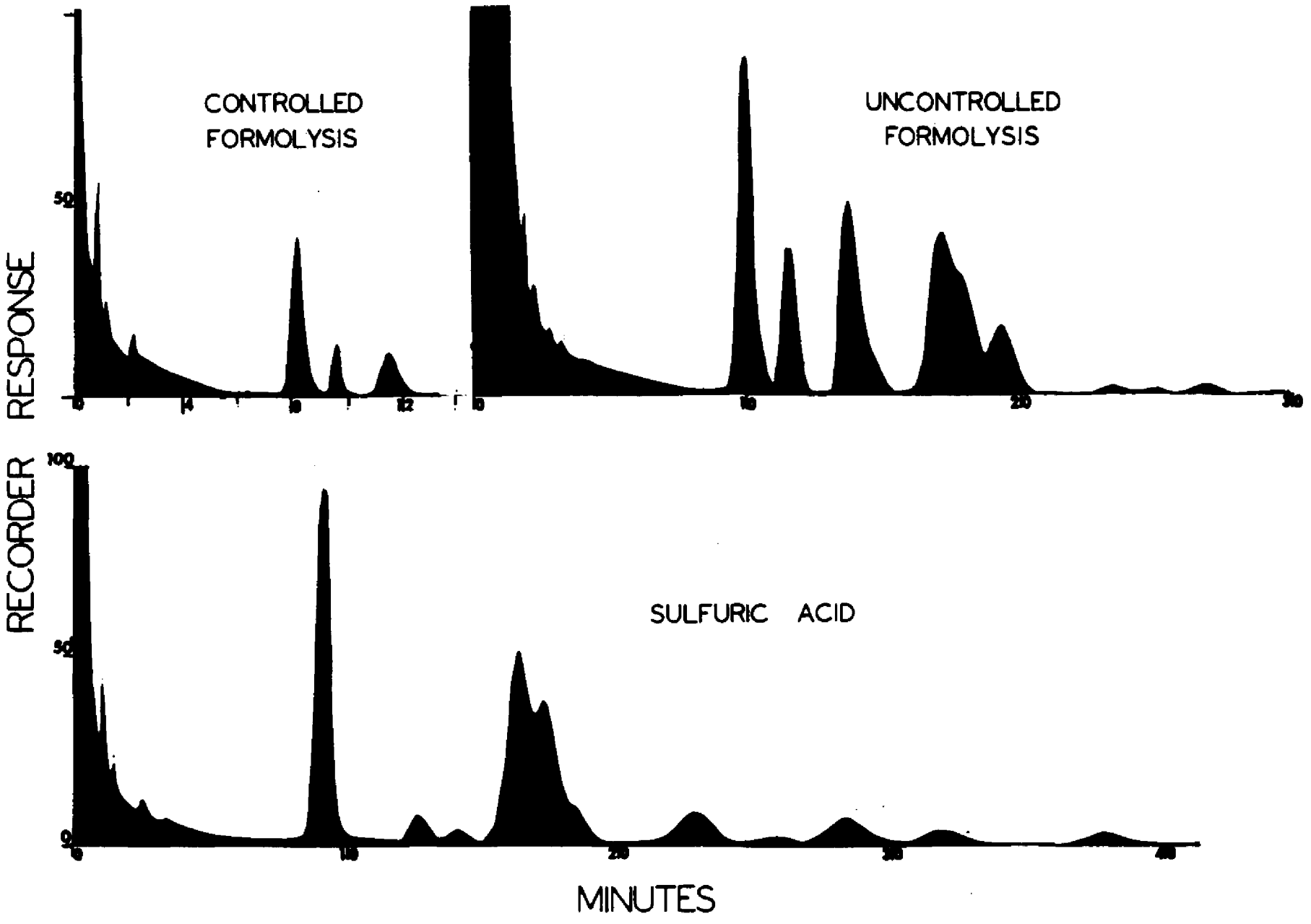

CONTROLLED
FORMOLYSIS
UNCONTROLLED
FORMOLYSIS
RECORDER RESPONSE
SULFURIC ACID
MINUTES

Figure 20. Gas Chromatogram (TMS Derivatives) of Methylated Rabbit Liver Glycogen Obtained by a Modification of the Formolysis Method.

RECORDER RESPONSE
0
50
100
4
8
12
16
20
24
28
MINUTES

Figure 21. Gas Chromatogram (TMS) Derivatives of the Hydrolytic Products Obtained by Hydrolysis of Octamethyl Maltose with 0.5N Sulfuric Acid.

RECORDER RESPONSE
MINUTES

demethylation and degradation by sulfuric acid was shown by the several peaks after tetramethylglucopyranoside.

The modification of the formolysis method was used to hydrolyze the methylated glycogen samples. Initially, neutralization was accomplished with a layered bed of Dowex 1 and Dowex 50 resins; later, deionization was carried out with Amberlite IR-4B. Recovery of standard (methyl-α-glucopyranoside) from the two types of columns was 94.6% and 92.8%, respectively, as determined by gas-liquid chromatography of the silanized glucoside. After hydrolysis, neutralization with the resins, and evaporation of the eluants under reduced pressure, the residues were taken up with 0.5 ml anhydrous pyridine. An aliquot of this solution was removed and chromatographed on thin-layers of silica gel G, using a modification of the paper chromatographic method of Hirst et al. (1949). The results of the thin-layer chromatography in n-butanol:ethanol:water:ammonia (40:10:49:1) are presented in Table 19 for the hydrolyzed methylated glycogens. The optical isomers of the methylated hexoses were not resolved by thin layer chromatography and each pair of isomers was observed as a single spot.

After aliquots of the pyridine solutions were chromatographed by thin-layer techniques and the methylated glucose derivatives identified, the remaining solutions were transferred to small glass vials and the methylated sugars were silanized by the method of Sweeley et al. (1963). The final reaction products, separated and determined quantitatively by the gas chromatograph were:

Table 19. Results of Thin Layer Chromatography of the Hydrolytic Products of Methylated Glycogens. [1/]

	Standards			Boll Weevil				Rabbit Liver Standard
	$(CH_3)_4$	$(CH_3)_3$	$(CH_3)_2$	c.w.e. eggs (#112)	c.w.e. eggs (#133)	c.w.e. larvae (#120)	TCAe. larvae (#130)	
$(CH_3)_4$ glucose	0.72			0.72	0.71	0.73	0.72	0.72
$(CH_3)_3$ glucose		0.66		0.64	0.65	0.67	0.66	0.66
$(CH_3)_2$ glucose			0.61	0.60	0.58	0.59	0.61	0.61

[1/] Silica gel G, water slurry.

[2/] n-Butanol:ethanol:water:ammonia (40:10:49:1); developed with 1% p-Anisidine in 10-ml methanol diluted to 100-ml with butanol.

2,3,4,6-tetra-O-methyl-1-O-trimethylsilyl-D-glucopyranoside (α- and/or β).

2,3,6-tri-O-methyl-1,4-di-O-trimethylsilyl-D-glucopyranoside(α- and/or β).

2,3-di-O-methyl-1,4,6-tri-O-trimethylsilyl-D-glucopyranoside(α- and/or β).

Early in the evaluation of the new method for polysaccharide structure determinations, a standard mixture of the TMS derivatives of tetra: tri:dimethylglucopyranoside was prepared. Initially, as shown in Figure 22, only one peak was detected for the tetramethyl (peak #1) and the trimethylglucopyranoside (peak #2), and two peaks (#3 and #4) for the dimethylglucopyranoside. Figure 22 shows the gas chromatogram of this same standard mixture after storage in the refrigerator (5°C) for 13 months. The quantity of β-dimethylglucopyranoside (#5) decreased more than 50% and a new peak (#3) appeared between the β-trimethyl and α-dimethylglucopyranosides.

In an attempt to explain the appearance of peak #3 (Figure 22b), a new standard mixture of TMS derivatives was prepared after allowing each sugar to stand overnight at room temperature in water (to allow mutarotation to α,β). Gas chromatography of the freshly prepared (mutarotated) standard mixture yielded data similar to that obtained for the 13 month old sample when it was chromatographed immediately after preparation. Assuming that these changes exhibited by the stored standard mixture would be accelerated at room temperature, the mutarotated standard mixture was allowed to stand (at room temperature) for 21 days; an aliquot was gas chromatographed every day or so. The resulting changes in the TMS derivatives are presented in Figure 23 for the storage times up to 21 days at room temperature. As previously

Figure 22. Gas Chromatograms of Trimethylsilyl Derivatives of Methylated Glucopyranosides Immediately Following Preparation (Standard Mixture, 1964) and (b) 13 Months After Preparation (5°C).

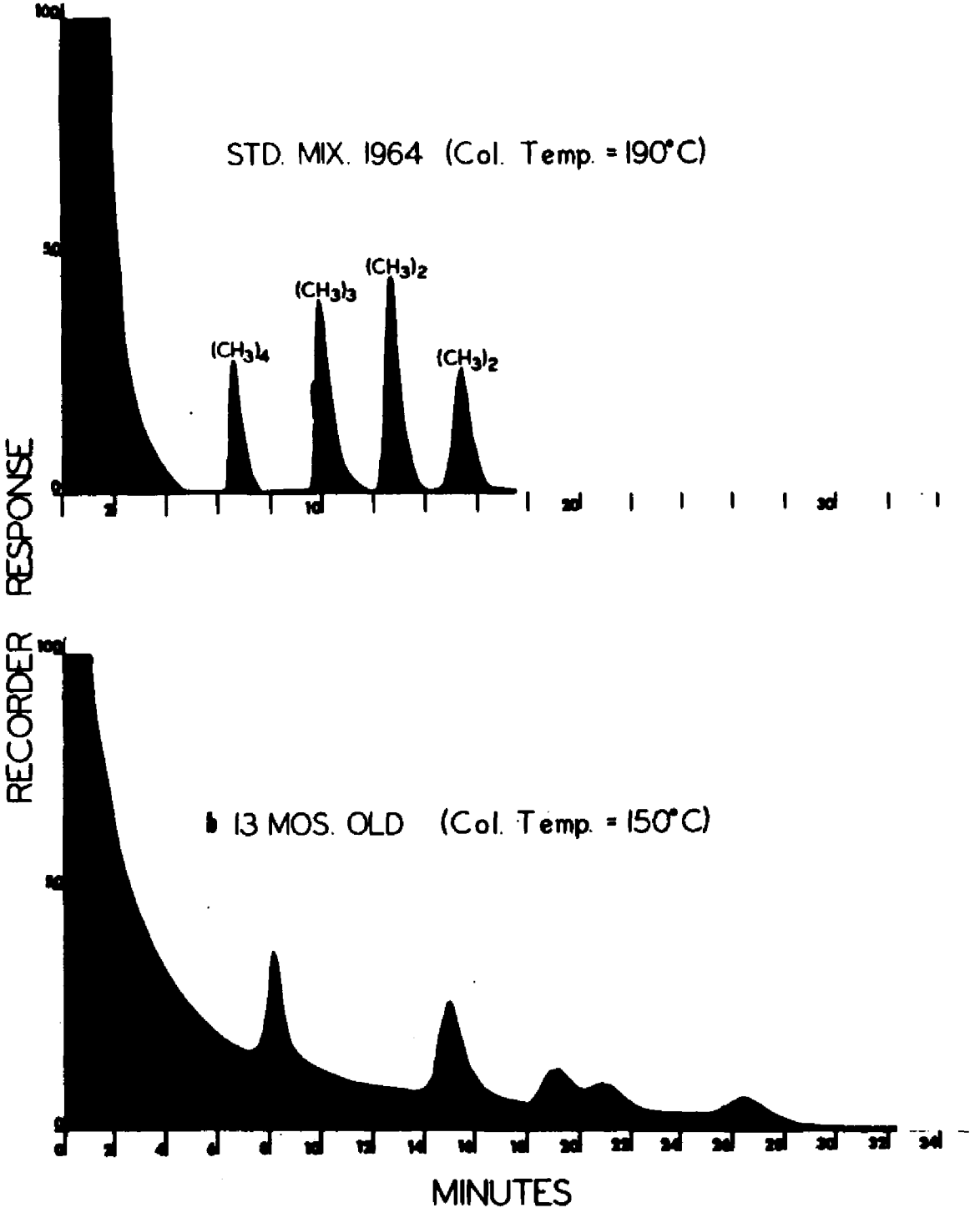
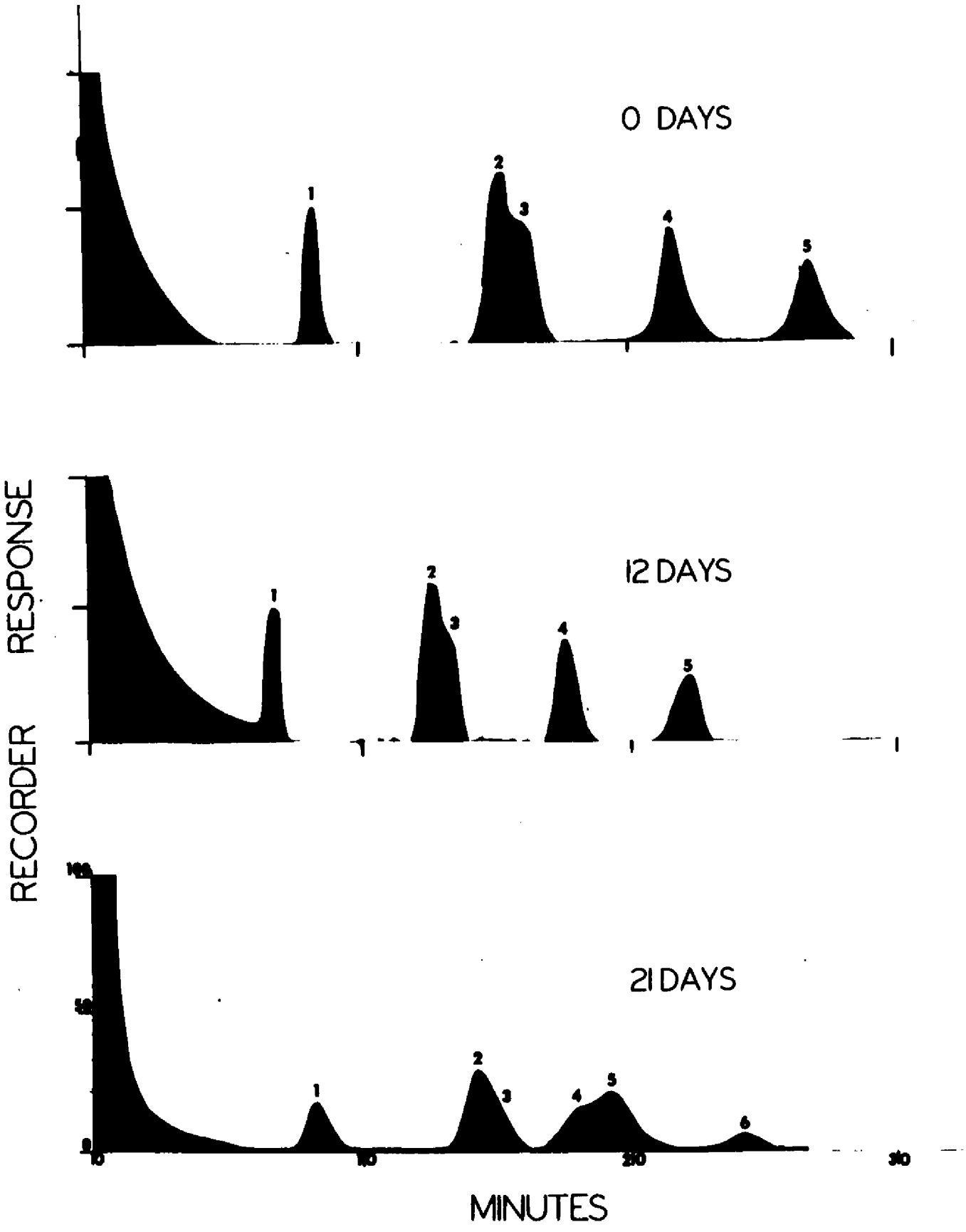

STD. MIX. 1964 (Col. Temp. = 190°C)
(CH3)4
(CH3)3
(CH3)2
(CH3)2
RESPONSE
RECORDER
b 13 MOS. OLD (Col. Temp. = 150°C)
MINUTES

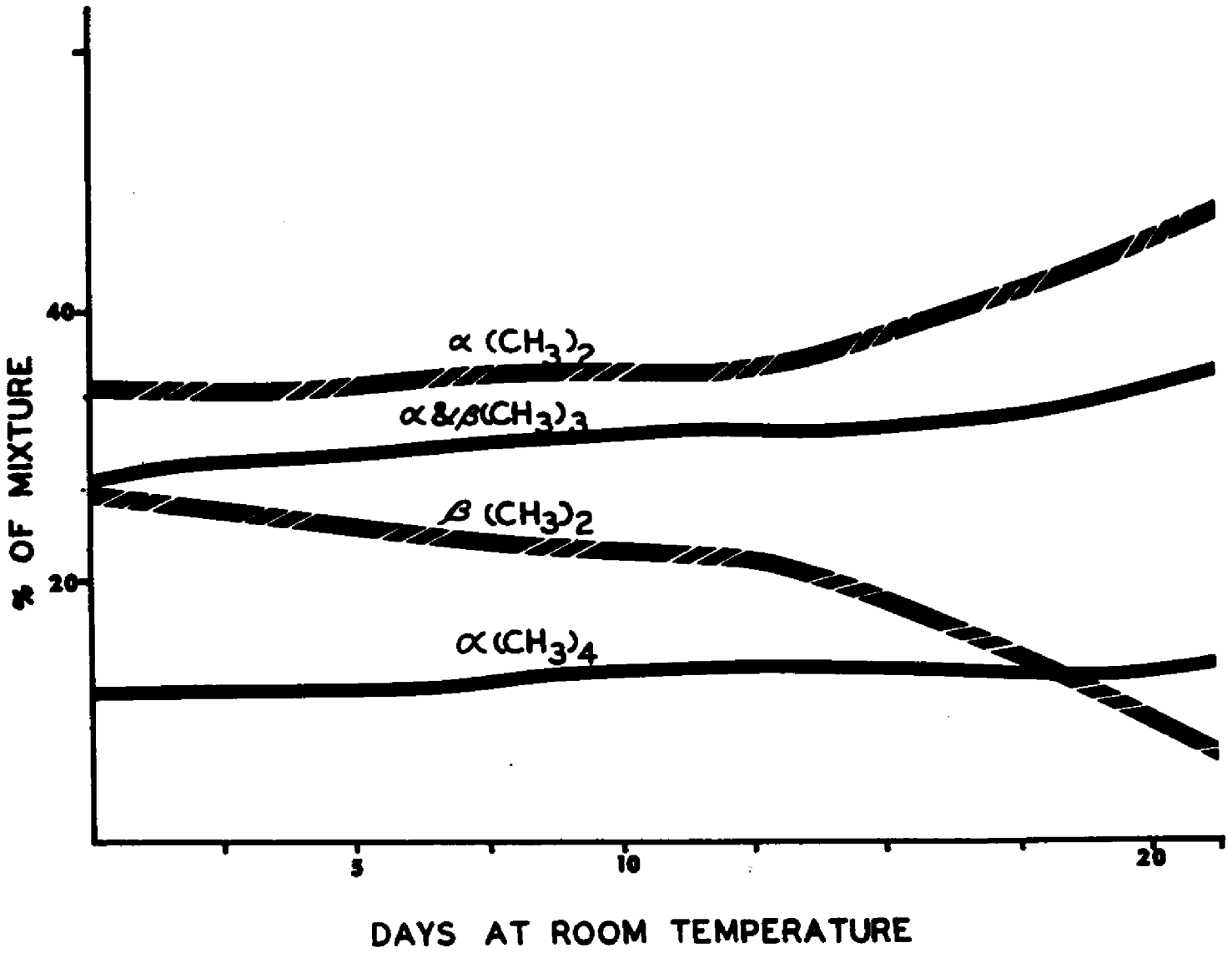
Figure 23. Changes in Trimethylsilyl Derivatives of Mutarotated Glucopyranosides at 0, 12, and 21 Days (Stored at Room Temperature).


0 DAYS
12 DAYS
21 DAYS
RECORDER RESPONSE
MINUTES
1
2
3
4
5
6

encountered with the refrigerated standard mixture, there was a decrease in the size of the β-dimethyl peak and a new peak appearing prior to the retention time for α-dimethylglucopyranose. Figure 24 is a graph of the changes occurring at room temperature and shows a 17% decrease in β-dimethyl during storage 21 days, and that the combined new peak (#4, 21 days) and the α-dimethyl peak (#5, 21 days) amounted to 17%. There were no significant changes in the composition of the tetra and trimethyl (TMS) derivatives.

Identification of the hydrolytic products of methylated boll weevil glycogen was primarily based on their respective retention times during gas-liquid chromatography. For this reason, gas chromatography was carried out on the individual methylated glucose standards before and after mutarotation. Freshly prepared, mutarotated 2,3,4,6-tetra-O-methyl-1-O-trimethylsilyl-α-D-glucopyranoside exhibited a major peak with retention time (t_r) = 10.0 min. and a very weak trace of the β-isomer (t_r = 13.5 min.). The β-isomer amounted to less than 1% of the sugar derivative, and apparently there is little mutarotation of tetramethylglucopyranose from its α-form. After allowing a water solution of 2,3,6-tri-O-methyl-α-D-glucopyranose to reach equilibrium, the TMS derivative was prepared and analyzed by gas-liquid chromatography. Although not well resolved by its gas chromatogram, the α- and β-isomers of this sugar were easily distinguished. The t_r's for the α-and β-isomers were 16.8 and 17.5 min., respectively, and no changes were observed during storage of the TMS derivative at room temperature for 21 days. The

Figure 24. Changes in Trimethylsilyl Derivatives of Mutarotated Methylated Glucopyranosides in a Standard Mixture Held at Room Temperature for 21 Days.


% OF MIXTURE
40
20
α (CH3)2
α&β(CH3)3
β (CH3)2
α(CH3)4
5
10
20
DAYS AT ROOM TEMPERATURE

equilibrium mixture of 2,3-di-O-methyl-1,4,6-tri-O-trimethylsilyl-D-glucopyranoside amounted to 38%α and 62% β. After storage of the sugar in the refrigerator 13 months, the β-peak decreased to 54% of the total, the α-peak increased to 39%, and the new peak (prior to α-dimethyl) became 7% of the total. These results paralleled those obtained for the dimethyl peaks in the stored standard mixture. However, freshly prepared, mutarotated dimethylglucopyranose did not change substantially during storage (alone) at room temperature for 21 days. The initial composition (56%α ; 44%β) was constant for 12 days and at the end of 21 days had changed to 59%α and 41% β. Retention times for the TMS derivatives of dimethylglucopyranose were 22 and 28 min. for the α- and β-isomers, respectively.

Some difficulties were encountered with the silanization of methylated rabbit liver glycogen (standard). The average chain length of rabbit liver glycogen (11 glucose units) yields, on methylation and hydrolysis, tetra:tri:dimethylglucopyranose in the ratio of 1:9:1. However, the theoretical ratio was observed only at specific times after silanization. During the evaporation of the deionized eluants the pale yellow liquid obtained could not be removed by evaporation at 30°C and reduced pressure, or by storage in a desiccator for three days. As a result, the silanization reaction was carried out with this pale yellow liquid rather than an anhydrous solution. It was found (unpublished data) that the silanization reaction must be moisture free, and storage of the pale yellow liquid in a desiccator in vacuo removed the liquid. Upon dissolution

of the residue with anhydrous pyridine, and reaction with the silanization reagents, the correct ratio was obtained consistently.

Results of the methylation, hydrolysis, and silanization analyses are presented in Table 20; the CL was compared for c.w.e. and TCAe. boll weevil samples and the standard glycogen. There was close agreement between the larval glycogens extracted by different procedures, indicating no structural damage (as such) to the molecular pattern by TCA.

There was close agreement between the new methylation method (Betz, Nettles, and Novak, unpublished data) and the enzymic assay as shown in Table 21. Enzymatic data were not obtained for two glycogen samples; however, it was apparent from the agreement between egg samples #112 and #133 that the methylation method gave reliable results. Although not duplicate samples (their storage conditions were different), they both were egg glycogen samples and methylation yielded 12.5 and 12.3 glycogen units for #112 and #133, respectively. These data are presented as their gas chromatograms in Figures 25, 26, and 27; the ratio obtained for each methylated glycogen sample is presented in Table 22.

The average exterior chain length (ECL) of boll weevil glycogens was obtained by two enzymic methods. The results (Table 18) of the phosphorylase limit dextrin determinations also yielded data characteristic of the ECL for these glycogens. The ECL was calculated from the per cent degradation of the sample by phosphorylase b alone and the average chain length (determined enzymically with phosphorylase b and amylo-1,6-glucosidase). The ECL by phosphorylase action,

Table 20. Results of the Average Chain Length Determinations of Glycogens Obtained by Three Extraction Procedures Upon Complete methylation, hydrolysis, silanization, and Gas-Liquid Chromatography.

	c.w.e.[1]	TCAe.[2]	KOHe.[3]
Average Chain Length	11.9	11.8	11.0

[1] Boll Weevil, larvae (#120).

[2] Boll Weevil, larvae (#130).

[3] Rabbit Liver, standard.

Table 21. Average Chain Lengths of Boll Weevil and Standard Glycogens by an Enzymic and a Non-enzymic Method (Methylation).

Sample	Average Chain Length[1]	
	Enzymic[2]	Methylation[3]
Boll Weevil, Eggs, c.w.e. (#112, MeOH)	12.5	11.0
Boll Weevil, Eggs, c.w.e. (#133, EtOH)	-	12.3
Boll Weevil, Larvae, c.w.e. (#120)	-	11.9
Boll Weevil, Larvae, TCAe. (#130)	12.4	11.8
Boll Weevil, Adults, c.w.e. (#109)	11.0	-
Rabbit Liver, Standard, KOHe.	11-18[4]	11.0

[1] Expressed as glucose units.

[2] Method of Bueding and Hawkins, 1964.

[3] Method of Betz, Nettles, and Novak, unpublished data.

[4] Kjølberg and Manners, 1962.

Figure 25. Gas Chromatograms of the Methylated, Hydrolyzed, and Silanized Derivatives of c.w.e. Egg Glycogen Samples Stored in Methanol and Ethanol.

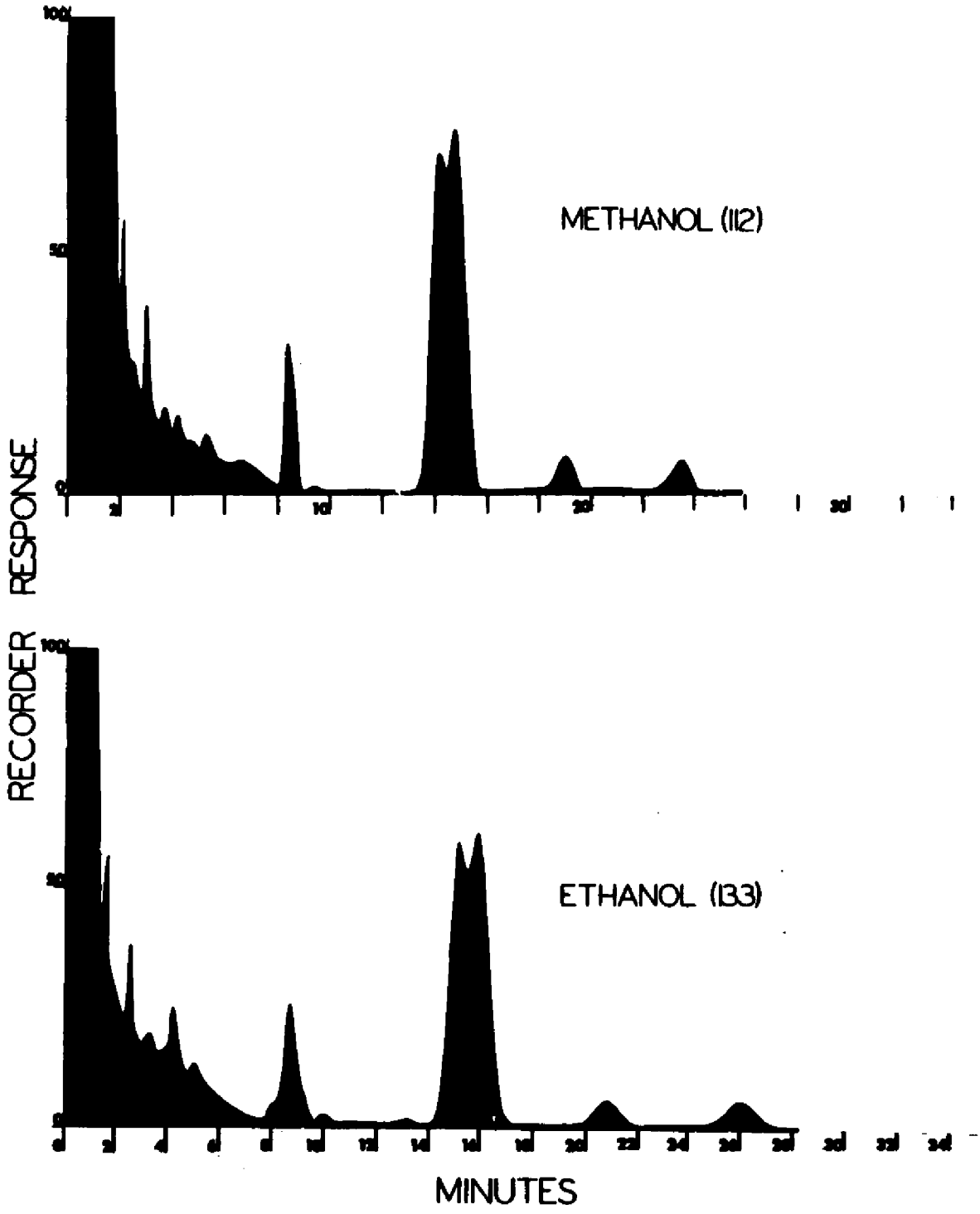

METHANOL (112)
ETHANOL (133)
RECORDER RESPONSE
MINUTES

Figure 26. Gas Chromatograms of the Methylated, Hydrolyzed, and Silanized Derivatives of c.w.e. Larval and TCAe. Larval Glycogens.

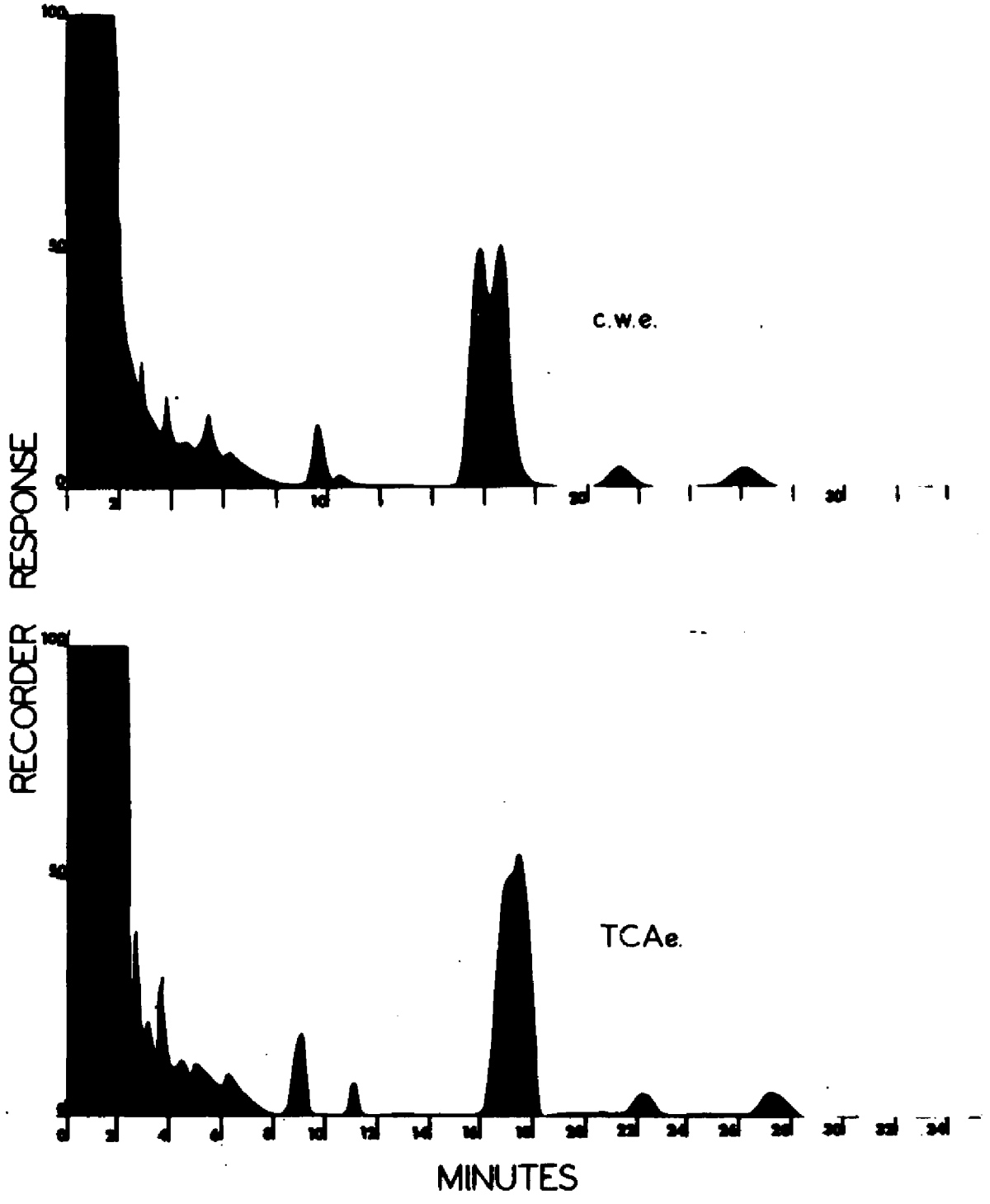

RECORDER RESPONSE
c.w.e.
TCAe.
MINUTES

Figure 27. Gas Chromatogram of the Methylated, Hydrolyzed, and Silanized Derivatives of KOHe. Rabbit Liver Glycogen.

RECORDER RESPONSE
MINUTES

Table 22. Ratio of Tetra:tri:dimethylglucopyranosides (TMS Derivatives) Obtained by Gas-liquid Chromatography of the Hydrolytic Products from Methylated Boll Weevil and Standard Glycogens.

	Sample				
	Boll Weevil				Rabbit Liver
	c.w.e. eggs #112	c.w.e. eggs #133	c.w.e. larvae #120	TCAe. larvae #130	Standard
$(CH_3)_4$ glucose	1.3	1.2	1.3	1.4	1.7
$(CH_3)_3$ glucose	8.7	10.1	9.6	9.3	8.3
$(CH_3)_2$ glucose	1.0	1.0	1.0	1.0	1.0

assuming that the limit of phosphorylase b is four glucose units from the branch points and is constant, are presented in Tables 17 and 18. The TCAe. larval glycogen had the largest exterior chain length of the boll weevil glycogens by phosphorylase assay.

The average exterior chain length was also calculated from the data obtained by incubation with β-amylase. These results, presented in Table 23, indicated larger average exterior chains than data yielded from other glycogens. The limit of β-amylase action, although not known exactly, has been estimated to be 1.0 - 2.5 glucose units removed from the triply branched glucose unit. The values presented in Table 23 are calculated from these accepted ranges for β-amylase.

The average interior chain length (ICL) was calculated for both enzymic assay methods from the relationship CL = ECL + ICL.

The major problem in determining the ICL according to all literature sources, is the size of the outer stub (from the α-1,6-linkage) after attack by phosphorylase b or β-amylase. These outer

Table 23. Average Exterior (ECL) and Interior Chain Lengths (ICL) of Boll Weevil and Standard Glycogens Based on the β-amylolysis Limit Dextrin.

Sample	% β-amylolysis	1/ CL	2/ ECL	3/ ICL
Boll Weevil, eggs, c.w.e. (#112, MeOH)	62.4	11.0	7.2 - 8.7	2.3 - 3.8
Boll Weevil, eggs, c.w.e. (#133, EtOH)	61.7	12.3	8.6 -10.1	2.2 - 3.7
Boll Weevil, larvae, c.w.e. (#120)	51.2	11.9	7.1 - 8.6	3.3 - 4.8
Boll Weevil, larvae, TCAe. (#130)	59.0	11.8	8.0 - 9.5	2.3 - 3.8
Rabbit Liver, Standard, KOHe.	52.4	11.0	6.8 - 8.3	2.7 - 4.2

1/Methylation.

2/ECL = glucose residues removed by β-amylolysis + (1.0-2.5).

3/ICL = CL - ECL.

stubs have been estimated to be 1-4 glucose units in length (not counting the triply branched glucose unit) following cleavage by phosphorylase b. Again, depending on the literature source, the average length of the outer stubs is 1.0-2.5 glucose units following β-amylase digestion. Therefore, the interior chain length varies with the length of the outer stub and this usually results in the expression of the ICL as a range of glucose units. The results of the ICL determinations, following phosphorylase digestion, are presented in Table 18. The ICL for boll weevil and standard glycogens by cleavage with β-amylase are presented in Table 23.

From the data yielded by methylation, hydrolysis, and silanization (a selective substitution of the free hydroxyl groups), the linkages were found to be primarily α. Hydrolysis produced approximately 82% of 2,3,6-tri-O-methyl-D-glucopyranose, indicating that at least 82% of the linkages are 1,4. Approximately 13% of the molecular structure yielded α-2,3,4,6-tetra-O-methyl-D-glucopyranoside indicating that all outer glucose units (attached only at the anomeric carbon atom) are of the α-configuration. It was found that 2,3-dimethylglucopyranose would freely mutarotate in an aqueous medium; no configurational assignment could be made for this triply branched glucose unit. However, the branched glucose unit in boll weevil glycogen was a 1,4,6-linked moiety.

DISCUSSION

The major objective of this study was the characterization of the physical and chemical properties and molecular structure of boll weevil glycogen. Glycogen was extracted with 5% trichloroacetic acid (TCA) and with cold chloroform-glycine buffer (c.w.e.) from eggs, larvae, and adult boll weevils.

During the early investigations of TCAe. boll weevil larval glycogen, a milder method of extraction was perfected by Bueding (personal communication). Bueding and Orrell (1965) reported that glycogen extracted by cold chloroform-glycine buffer exhibited high molecular weight components and that "native" glycogen can be obtained only by the milder c.w.e. method. Glycogen was cold-water extracted from boll weevil larvae and later from eggs and adults; the carbohydrate content was consistently in excess of 95%. According to Bueding and Orrell (1965), less product may be obtained by c.w.e. (because of the manipulations in the method), but it does not contain, preferentially, either the high or low molecular weight material. For this reason, the extracted glycogen approaches more closely the state of glycogen in the living cell. Although most data were in close agreement between TCAe. and c.w.e. samples, the results of this work confirmed that cold-water extraction is a necessity in structural analysis (i.e., molecular weight data obtained for TCAe. and c.w.e. glycogen were strikingly different).

Currently, the c.w.e. glycogens seem to approach more closely the form of glycogen *in vivo*; however, some analyses are prohibited by cold-water extracted material. For example, the optical rotation of 0.1% aqueous solutions cannot be determined because of the characteristic, but exceedingly high, opalescense. Although c.w.e. glycogens are stable at room temperature for indefinite periods, they appear to be difficult to solubilize (probably because of the higher molecular weight component). Obviously, c.w.e. glycogens could not be methylated with procedures that employ alkali or acid without severe degradation of the higher molecular weight component. Methylation reactions have been performed successfully on polysaccharides by many workers (Barker *et al.*, 1950; Van Cleve *et al.*, 1956; Bishop *et al.*, 1960; Bishop and Cooper, 1960) who employed alternate additions of sodium hydroxide and dimethyl sulfate to an aqueous solution. Hirst *et al.*, (1949) reported the successful methylation of glycogen by exposure to 4% methanolic hydrogen chloride at 100°C.

Whole weevil extracts were subjected to complete physical and chemical analyses; the component monosaccharide was identified, as was its chain length and linkage in the glycogen molecule.

Complete characterization of the extracted materials included tests to determine the per cent composition of each glycogen sample. The TCAe. larval glycogen was only 66% carbohydrate as determined by the anthrone method of Carroll *et al.* (1956), sulfuric acid method of Mendel *et al.* (1954), and analysis by glucose oxidase. Therefore, additional analyses were performed to further define the

TCAe. larval sample. The protein content (6.3%) of the TCAe. larval glycogen sample was determined by two methods with close agreement. This value was higher than that reported for any other glycogens (literature values are less than 1%; Good et al. (1933)). Moisture content was determined for boll weevil larval glycogen (TCAe.) and the result was 10.4%. The moisture content for a commercial (rabbit liver) glycogen was 10.4% indicating that the moisture probably resulted, at least in part, from handling. Ash content of the TCAe. sample was 7.7%. The rabbit liver sample, similarly treated, was ash-free. It was suspected that some ash of this boll weevil sample resulted from the large volumes of undistilled reagents used in its preparation.

The optical rotation of TCAe. larval glycogen, determined with the D-line of sodium, was $+200.0^{\circ}$. The optical rotation of other glycogens ranges from $+173^{\circ}$ to $+233^{\circ}$ (Manners, 1957) and the optical rotation of insect glycogens ranges from $+184^{\circ}$ to $+194^{\circ}$ (Table 1). It was found that TCAe. boll weevil larval glycogen had the highest optical rotation yet determined for insect glycogens. Measurements of the optical rotation of c.w.e. boll weevil glycogens were attempted, but concentrations of less than 1% were highly opalescent and interference prevented the measurement of specific rotation.

The iodine binding power of boll weevil glycogens was investigated in a solution of iodine and potassium iodide. The region of maximum absorption for rabbit liver glycogens was reported as 460mμ (Manners, 1962) and the observed absorption of the commercial sample

was 370 and 375mμ by determinations with the Beckman DU and recording DB spectrophotometers, respectively. The absorption of boll weevil TCAe. larval glycogen was 370 and 383mμ by the two methods. Regions of maximum absorption of the iodine-iodide complexes for the c.w.e. samples were 395mμ for the larval and adult samples and 400mμ for the c.w.e. eggs. Archibald et al. (1961) reported that a relationship exists between the iodine absorption powers of polysaccharides and their respective interior chain lengths. Their observations have contributed to the differentiation of branched polysaccharides based on the region of maximum absorption with an iodine-iodide solution. According to Archibald et al. (1961), and others (a review by Manners, 1957), polysaccharides exhibiting maximum absorption around 400mμ have an average interior chain length of less than five glucose units; those polysaccharides of maximum absorption greater than 500mμ have an average interior chain length greater than seven glucose units. These results represent a major distinction between glycogen (400mμ) and amylopectin (600mμ). The results of interior chain length determinations on boll weevil and standard glycogens support the theory of Archibald et al. (1961); the interior chain averaged 3.0 - 3.5 glucose units.

Infra-red spectra from TCAe. and c.w.e. boll weevil larvae were identical to the spectrum obtained for rabbit liver glycogen. The spectra showed all regions of absorption characteristic of α-glucopyranoses and hydroxy compounds. The Sadtler index was 94---47889-2-98 and 94---57889-2-98 for rabbit liver and boll weevil

glycogens (TCAe. and c.w.e.), respectively. The Sadtler code (Sadtler Index of Scientific Information) reported in the literature was 94---5788--2-98. All samples showed a region of infra-red absorption around 7.5 microns that was not identified.

Molecular weight data were obtained for TCAe. larval and c.w.e. egg, larval, and adult boll weevil glycogen. The sedimentation data for the c.w.e. samples, with the exception of the egg glycogen, corresponded closely with data collected for c.w.e. Ascaris, Fasciola hepatica, and Hymenolepis diminuta (Orrell et al., 1964). The c.w.e. sedimentation patterns exhibited two distinct regions of molecular weight; the higher molecular weight material was continuous with the lower molecular weight material. Apparently, an increase in the sugar (as sucrose) content of the larval diet failed to influence either region of molecular weight. Sedimentation data collected for c.w.e. larvae on a high sucrose diet (20%) did not differ substantially from the data obtained for the normal diet B. Sedimentation data were also attempted for c.w.e. adults, fed 14 days on different diets (squares or bolls). Prints were obtained from the Schlieren optical system for both glycogens; however, the IBM system failed to correct the data yielded by glycogen from the square-fed adults (i.e., concentration was too low). The sedimentation pattern obtained for boll-fed adults was similar to that obtained for the other c.w.e. materials except that the two molecular weight regions had higher weight averages than those exhibited by c.w.e. larval glycogen. Although the two regions of molecular weight were quantitatively

less than the other c.w.e. glycogens, the weight averages were the highest obtained for boll weevil glycogens with the exception of c.w.e. egg glycogen.

Cold-water extracted boll weevil egg glycogen had a higher average molecular weight (based on sedimentation prints alone) than all other naturally occurring polysaccharides analyzed to date.

Attempts were made to support or invalidate the sedimentation data by two additional cold-water extractions of boll weevil egg glycogen. Upon sedimentation analysis, these additional samples confirmed the data obtained for the first sample (i.e., the same sedimentation pattern was obtained).

The results of this study of sedimentation patterns, at first, seem in opposition to the space-limited disk structure for glycogen, proposed by Madsen and Cori (1958) and later supported by French (1964). The difficulty, presented by the exceedingly heavy molecular weight fraction of egg glycogen, is evidenced by attempting to construct a physical model for glycogen of molecular weight greater than 20×10^6. According to French (1964), from the reducing end a model can be constructed to a point at which the periphery is so densely packed that it becomes sterically impossible to continue regular branching. The total number of glucose units which can be accommodated by such a structure would be about 122,000 (molecular weight 20×10^6). The space-limited structure probably does exist and the heavy molecular weight material can be attributed to aggregates of the smaller (space-limited) structure.

Digestion of boll weevil glycogens by β-amylase resulted in β-amylolysis limit dextrins smaller than those defined to date. The per cent cleavage by three times recrystallized (sweet potato) β-amylase was greater than rabbit liver glycogen (45 ± 5%; Manners, 1952), and in some samples greater than that obtained by amylopectin digestion with the enzyme (55 ± 5%; Manners, 1952). This study represented the first isolation and identification of maltose from digestion by β-amylase using Sephadex gel. The successful separation of maltose from the limit dextrin (L.D.) has previously been accomplished by precipitation with ethanol. However, the isolation of maltose from the L.D. by Sephadex gel has now been successfully performed on the enzymic digest after cleavage by β-amylase.

The results obtained with this enzymic assay indicated longer exterior chains in boll weevil glycogen than those contained in rabbit liver glycogen. The largest exterior chain difference was two glucose units between c.w.e. egg glycogen (9.4 units) and the standard (7.6 units). The per cent β-amylolysis for this sample averaged 61.7. The closest value to the standard (52.4%) of β-amylase action on boll weevil glycogen was obtained for c.w.e. larval glycogen (51.2%). The average exterior chain length for boll weevil glycogens was calculated from the β-amylolysis data. The respective chain lengths were expressed as a range since the termination of action of β-amylase is not known with certainty (i.e., 1.0 - 2.5 units removed from the triply branched glucose unit). As a result, the average interior chain lengths could be reported only as a range.

Phosphoryolysis was also performed on the boll weevil glycogens to determine the phosphorylase limit dextrin and the average chain length. Values obtained for digestion with phosphorylase alone varied between 26.5 - 39.6%; values reported in the literature range from 20 - 30% (Manners, 1957) depending on the source of the phosphorylase. The exterior chain lengths of these glycogens were calculated from data obtained by phosphorylolysis and the limit of phosphorylase action on glycogen. Currently, the theory most widely accepted is that of Walker and Whelan (1960): the limit of phosphorylase b action is four glucose units removed from the outer branch.

The results of enzymic degradation, by β-amylase and phosphorylase b, were in close agreement for the rabbit liver glycogen sample. The average exterior and interior chain lengths by β-amylase assay were 7.6 and 3.4 glucose units, respectively; phosphorylase b degradation of the same sample gave an exterior chain length of 7.5 glucose units and an interior chain length of 3.5. However, assuming the action of these two enzymic preparations is constant, conflicting data were obtained by the two methods. The longest exterior chain length for boll weevil glycogen obtained by β-amylase was egg glycogen (c.w.e.) and by phosphorylase action was TCAe. larval glycogen. But from data yielded for the exterior chain length of these two glycogens, the average exterior chain lengths only differed by 0.3 glucose unit for the c.w.e. sample and 0.1 glucose unit for the TCAe. sample. If it is correct to assume that the action of these enzymes is constant, the exposure of the outer chains (of a possible space-limited disk

structure) may sterically protect part of the exterior chain from cleavage by one of these enzymes. It has been shown, however, that neither β-amylase nor phosphorylase b action is constant (Manners, 1957).

Classical methods for the determination of the average chain length of polysaccharides have depended largely on methylation, hydrolysis, and identification of the hydrolytic products. Indications of the probable value of methylation as an analytical tool for structural work appeared in the early literature. Purdie and Irvine (1903) successfully methylated sugars with a mixture of silver oxide and alkyl (most often methyl) iodide. Oligosaccharides, after methylation, were acid hydrolyzed with dilute hydrochloric acid followed by distillation. Haworth (1915) modified the methylation technique by using methyl sulfate, dissolved in aqueous sodium hydroxide, as the methylating reagent. Despite the many references on methylation, Hirst and Percival (1965), have reported the impossibility of completely methylating glycogen by single treatment with methylation agents. A combination of methylglucoside formation and methyl ether formation, requiring different methylation reagents, was necessary. The methylation technique, developed during the present study, probably yields a fully-methylated glycogen molecule (non-polar) since differential solubility with chloroform (non-polar) was employed. Although it is possible that all glycogen molecules were not fully methylated, certainly the chloroform-extracted glycogen was saturated with methoxy groups.

Determinations of the methylated hydrolytic products from early works employed fractional distillation; later paper

chromatographic techniques were developed (Hirst et al., 1949; Bartlett et al., 1951), and most recently gas-liquid chromatographic methods have been successful (Kircher, 1960; Bishop et al., 1960; Bishop and Cooper, 1960).

Methylated sugar mixtures have been separated successfully by gas-liquid chromatography (reviewed by Kircher, 1960), but since anomers and isomers of methylated sugars are separated and detected, the chromatograms are sometimes difficult to analyze.

However, the results of this study show that polysaccharides can be methylated directly and their hydrolytic products determined by gas-liquid chromatography. It was found that to successfully determine both qualitatively and quantitatively the released methylated sugars from fully methylated glycogen, the silanization reaction must be performed under moisture-free conditions. Changes occurred in both the dimethyl-and-trimethylglucopyranosides following the exposure of methylated hydrolytic products (containing some water) to the silanization reactants. The theoretical ratio (from chain length) was obtained briefly, between 0.5 - 5.0 hours, after silanization of a methylated rabbit liver glycogen standard. The average chain lengths for the boll weevil glycogens, when prepared in a moisture-free environment, were in close agreement with the average chain lengths determined by the enzymic system. The chain lengths were reproducible for 18 hours after preparation of the trimethylsilyl derivatives; the samples were stored in a desiccator over phosphorus pentoxide to protect them from moisture.

An additional problem was resolved during the work with the methylated-silanized hexoses. Often, while working with small amounts of starting material, it was necessary to evaporate the pyridine solutions to obtain gas-chromatographic data. It was found, during this study, that the sequence of methylation, hydrolysis, deionization, evaporation, and silanization must be rigidly followed. Once the trimethylsilyl derivatives were formed, any evaporation of the solvent and reactants resulted in gas chromatograms that could not be reproduced.

An artifact was produced by the hydrolytic method (formolysis) used in this study, but was completely eliminated by modifying the temperature and time of exposure to the formic acid. The formic-sulfuric acid hydrolysis caused less degradation to the methylated glycogen with no detectible demethylation and agrees with an observation by Croon et al. (1960).

The triply branched glucose units in boll weevil glycogen were determined to be linked 1, 4, 6, since the dimethyl sugar was identified as 2, 3-dimethylglucopyranose. Because of the inherent difficulties in maintaining anhydrous conditions during acid hydrolysis, both α- and β-peaks were obtained. Therefore, it was impossible to assign a configuration to the anomeric carbon atom of di- and trimethylglycopyranose. However, only α-tetramethylglucopyranose was detected and since β-tetramethyl cannot be obtained by mutarotation, the configuration of the anomeric carbon atom of all peripheral glucose units was determined to be α .

It was also evident during this study, that no isomerization or mutarotation takes place as the methylated-silanized derivatives. There was no difference in the relative amounts of α- and β-isomers of the individual methylated sugars after standing either for 13 months at 5°C or for 21 days at room temperature.

Other than the high molecular weights exhibited by the c.w.e. glycogens, the physical properties of the c.w.e. and TCAe. boll weevil glycogens were comparable to the rabbit liver standard. However, the average exterior chain lengths for boll weevil glycogens were larger than the exterior chain lengths for other glycogens, as shown by their per cent β-amylolysis, by enzymic assay, and by methylation and gas-liquid chromatography. The average chain lengths of all the boll weevil samples were $\pm$1 glucose unit of the standard glycogen. A methylation technique was successfully used during this study to obtain the average chain lengths and chain linkage configurations of the component monosaccharide, glucose.

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VITA

Norman Leo Betz was born on January 23, 1938 in Baton Rouge, Louisiana. After attending schools in Baton Rouge, he received his high school diploma from Saint Joseph's Academy in New Roads, Louisiana in 1956. He entered Louisiana State University in the fall of that year, and during the fall of 1957 he accepted a research position with the Department of Zoology. Upon receipt of the Bachelor of Arts degree in 1961, he was granted the junior research fellowship with the Louisiana Petroleum Refiners' Waste Control Council. In January, 1962, he was granted the senior fellowship to pursue the Master of Science degree, which he received from the Department of Food Science and Technology in June 1963.

He accepted a research position with the United States Department of Agriculture, Entomology Research Division, Baton Rouge, Louisiana in July 1962,where he is presently employed. He is now a candidate for the degree of Doctor of Philosophy in the Department of Food Science and Technology.

EXAMINATION AND THESIS REPORT

Candidate: Norman Leo Betz

Major Field: Food Science and Technology

Title of Thesis: Physicochemical Properties and Molecular Structure Determination of Glycogen From Anthonomus grandis Boheman.

Approved:

Arthur J. Novak
Major Professor and Chairman

Max Goodrich
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Date of Examination:

December 17, 1965